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14. ABSTRACT
The estrogen receptor (ER) is implicated in the progression of breast cancer. Despite positive effects of hormonal therapy, initial or acquired resistance to endocrine therapies frequently occurs. To establish the significance of ER-Src axis in PELP1 and HER2 mediated therapy resistance, we have generated model cells that stably express Src-shRNA under conditions of PELP1, HER2 deregulation. Depletion of Src using shRNA substantially reduced E2 mediated activation of Src and MAPK activation in resistant model cells. Pharmacological inhibition of Src using dasatinib, an orally available inhibitor substantially inhibited the growth of therapy resistant MCF7-PELP1, MCF7-HER2, and MCF7-Tam model cells in proliferation assays. In post-menopausal xenograft based studies, treatment with dasatinib significantly inhibited the growth of therapy resistant cells. Since PELP1, HER2, and Src kinase are commonly deregulated in breast cancers, combination therapies using both endocrine agents and dasatinib may have better therapeutic effect by delaying the development of hormonal resistance.

15. SUBJECT TERMS
Estrogen receptor, coregulators, nongenomic actions, Src kinase, therapy resistance, metastasis

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Annual Report

Award Number: W81XWH-08-1-0604
Project Period: September 1, 2008 – August 31, 2011: No cost extension August 2012
Title: Hormonal Resistance and Metastasis: ER-coregulator-Src Targeted therapy
PI: Ratna K Vadlamudi
Report Period: September 1, 2010 – August 31, 2011

INTRODUCTION:

The estrogen receptor (ER), is implicated in the progression of breast cancer (1). Endocrine therapy using Tamoxifen, a selective estrogen receptor modulator (SERM), has been shown to improve relapse-free and overall survival (2). More recently, aromatase inhibitors, which deplete peripheral estrogen (E2) synthesis, are shown to substantially improve disease-free survival in postmenopausal women (3). Furthermore, endocrine therapy also shown to have a positive effect on the treatment of advanced metastatic disease. Despite these positive effects, initial or acquired resistance to endocrine therapies frequently occurs. *Accumulating evidence suggests that ER-coregulators play an essential role in hormonal responsiveness and cancer progression* (4-6). Proline, Glutamic-acid and Leucine-rich Protein 1 (PELP1) is a recently identified novel ER coregulator (7, 8). Emerging evidence suggests that ER signaling cross talk with growth factors play an important role in hormonal resistance and metastasis. Since multiple signaling pathways in addition to hormone are involved in activating ERs, **combination therapies** using both endocrine and nonendocrine agents that block different pathways may have **better therapeutic effect** and may delay development of hormonal resistance and metastasis. Recent evidence implicates ER-coregulator PELP1 play an essential role in coupling ER with Src kinases leading hormonal resistance. *In this study, we hypothesize that deregulation of PELP1 promotes Src activation and excessive signaling crosstalk with ER, leading to hormonal therapy resistance and metastasis.* This proposal is aimed to determine whether PELP1-Src signaling is a rate limiting factor in the development of hormonal independence and metastasis and to test whether blocking of the PELP1-Src pathway in combination with endocrine therapies prevent hormonal therapy resistance and metastasis.

BODY:

The scope of this proposal is to undertake the following two tasks outlined in the approved statement of work:

Task 1. **To establish the significance of ER-coregulator-Src axis in hormonal resistance and metastasis**

Task2. **To determine the efficacy of targeting of the ER-coregulator-Src axis on hormonal therapy and metastasis**

As per the recommendation given in the summary of 2^{nd} year report, we have included below only experimental data that was generated during the third year of this study. However, we summarized the key findings for all three years at the end in bullet form.

Dasatinib decreases ER-coregulator PELP1 oncogenic potential *in vivo*: PELP1 deregulation promotes *in vivo* tumorigensis (9). PELP1 also promotes local estrogen synthesis via Src kinase pathway facilitating growth of tumors in an autocrine manner (10). Therefore, we hypothesized that PELP1 driven tumors can be therapeutically targeted using dasatinib (a src kinase inhibitor) and tested using a postmenopausal xenograft model. Nude mice (nu/nu) were injected with control MCF7 cells or MCF7-PELP1 cells that overexpress PELP1 by mixing them with equal volume of Matrigel. Because athymic mice were deficient in adrenal androgens, they were supplemented daily with s.c. injections of the aromatase substrate androstenedione (100 µg/d) for the duration of the experiment. Under these conditions, injected MCF7 cells did not form tumors. As observed before, MCF7-PELP1 expressing cells formed tumors in the absence of exogenous estrogen supplementation suggesting local derived estrogen supported the growth of MCF7-PELP1 cells. When the tumor volume reached 100 mm^3, mice were either treated with dasatinib (15mg/kg/day/*oral*) or treated with vehicle (citrate buffer). Dasatinib treatment significantly reduced the PELP1-driven tumor volume (Fig. 1a) and tumor weight (Fig. 1b). Dasatinib treated tumors revealed decreased proliferation as evidenced by decreased nuclear PCNA staining and exhibited decreased Src and MAPK kinase activity seen by diminished phospho antibody staining (Fig. 1c). These results suggested that functional Src-MAPK axis is needed for PELP1 mediated tumorigenesis *in vivo* and dasatinib can be potentially used to reduce ER-coregulator PELP1 mediated tumor growth.

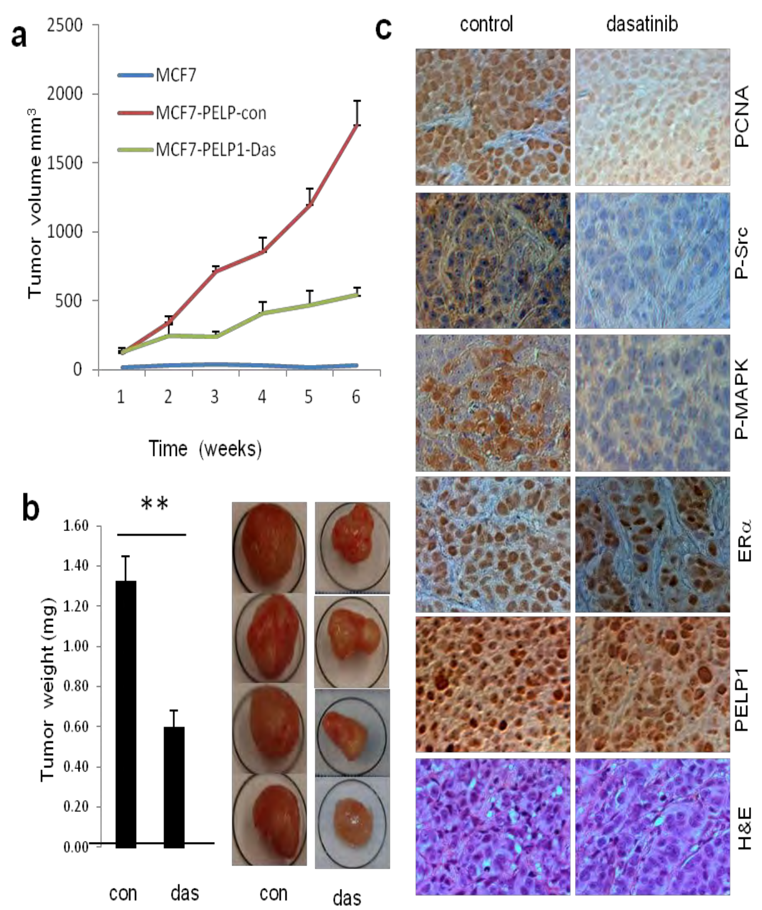

Figure 1. Dasatinib reduces PELP1 driven cancer progression *in vivo*. **a**, Nude mice were injected subcutaneously with MCF7 or MCF7-PELP1-WT. When the tumor reached ~100 mm3, it was treated with or without dasatinib. Tumor growth was measured at weekly intervals. Tumor volume is shown in the graph. **b**, The average tumor weight is shown in the graph. Representative images of tumors are shown. **c**, Status of PELP1, ER, Src phosphorylation and PCNA expression as a marker of proliferation was analyzed by immunohistochemistry (IHC). *, P<0.05.

Effect of Dasatinib combination on the growth therapy resistant cells *in vivo*: PELP1 deregulation is known to promote tamoxifen resistance (11, 12). We therefore examined whether dasatinib reduces PELP1-mediated therapy resistance. Xenografts established as described in fig 1, were randomly assigned to groups and treated with tamoxifen, letrozole, dasatinib alone or in combination. As seen in previously published studies (11), tamoxifen did not affected the growth of PELP1 driven tumors, while dasatinib substantially inhibited tumor volume (Fig. 2a) and weight (Fig. 2b). Combination treatment of tamoxifen with dasatinib showed further decreases in tumor volume and growth compared dasatinib alone however the differences are not statistically significant. Similarly, letrozole, an aromatase inhibitor, substantially reduced PELP1 driven tumor growth underscoring the importance of locally synthesized estrogen in PELP1 driven tumor growth. However the combination of letrozole with dasatinb only slightly enhanced the therapy response. IHC examination of PCNA staining revealed that dasatinib treatment decreased proliferation of tumor cells (Fig. 2c), while TUNEL staining showed increased apoptosis in dasatinb treated tumor cells (Fig. 2d). Collectively, these results suggest that pharmacological inhibition of Src could be used to treat therapy resistance induced by deregulation of proto-oncogene PELP1.

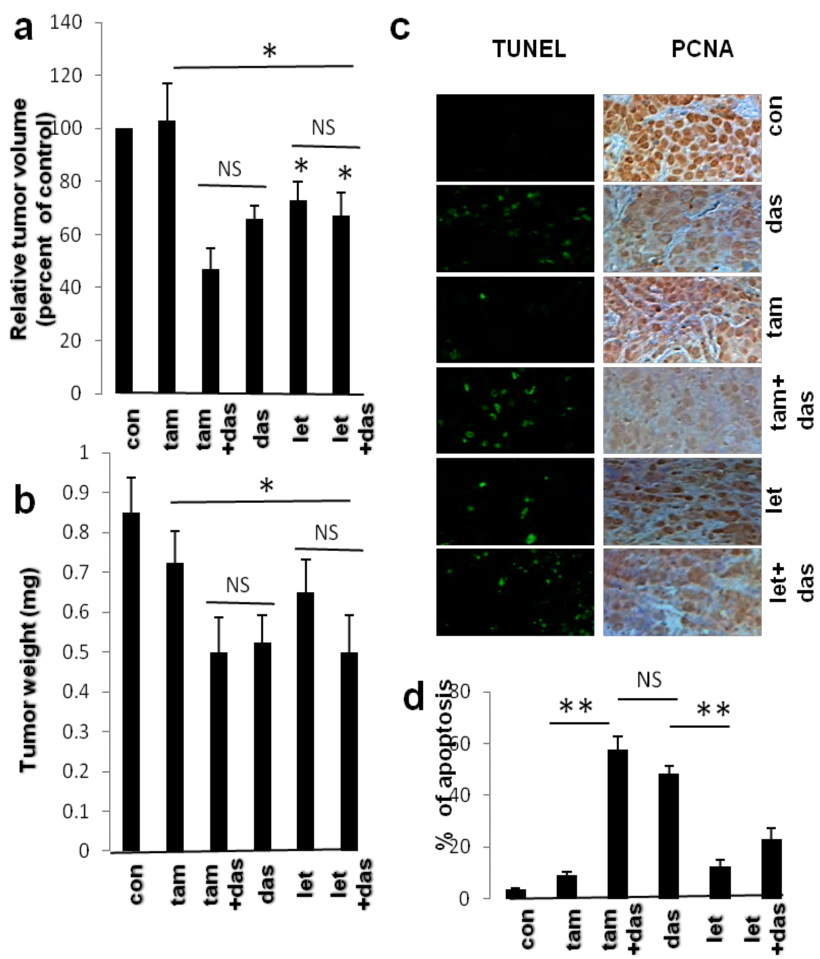

Figure 2. Antitumor effects of dasatinib in combination with tamoxifen and letrozole. **a**, Nude mice were injected subcutaneously with MCF7-PELP1-WT. When the tumor volume reached ~100 mm3, then dasatinb or combination treatment was started and tumor growth was measured at weekly intervals. Tumor volume is shown in the graph. ***b***, The average tumor weight is shown in the graph. **c**, TUNEL staining as a marker of apoptosis and PCNA expression as a marker of proliferation was analyzed by immunohistochemistry (IHC). *, P<0.05. d, Representative results and quantitative analysis of TUNEL staining. Columns, mean TUNEL labeling percentage based on five randomly selected high-power microscopic fields for each group. **,P<0.001. NS, non significant.

PELP1 knock down via siRNA inhibits the growth of therapy resistant cells. Since Src inhibitor dasatinib reduced the growth of therapy resistant cells, we have hypothesized that down regulation of PELP1 via siRNA will also mimic the same effects and such finding will further implicate PELP1-Src axis play role in therapy resistance. To test this, we have used MCF7-TAM that exhibit tamoxifen resistance, MCF7-HER2 cells that overexpress oncogene HER2 and show Tamoxifen resistance, MCF-LTLT cells that acquired resistance to Letrozole. Model cells were treated with control or PELP1 specific siRNA nanoparticles (200 nM) for 72 h and the cell viability was determined using Cell Titer-Glo Luminescent Cell Viability Assay. PELP1 siRNA substantially inhibited viability of all the three model cells. These results further support the findings from the second year that PELP1-Src axis has potential to contribute therapy resistance and PELP1 siRNA nanoparticles can be used as a potential drug.

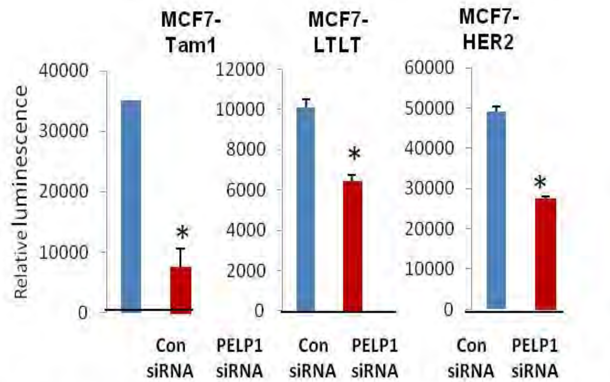

Fig 3. Efficacy of PELP1-siRNA nano-particles on various endocrine therapy resistant cell lines tested by viability assays.

PELP1 knockdown reduces proliferation of ER-negative breast cancer cells: Since expression of both PELP1 and Src is also maintained in ER-ve tumors, we examined whether PELP1-Src axis also play a role in growth of ER-ve breast cancer cells. We used two ER-negative model cells: MDA-MB231 (human) and 4T1 (mouse). Earlier studies showed that these models cells metastasize efficiently to sites affected in human breast cancer (13, 14) and both cells express high levels of PELP1. To establish the significance of the PELP1 axis, we knocked down PELP1 expression using lentiviral-mediated transduction of PELP1-shRNA. Pooled clones stably expressing PELP1-shRNA were selected by puromycin. qRTPCR and Western analysis showed that PELP1 expression in MDA-MB231-PELP1shRNA and 4T1-PELP1shRNA model cells was reduced by 70-80% (Fig. 4A). We next examined whether

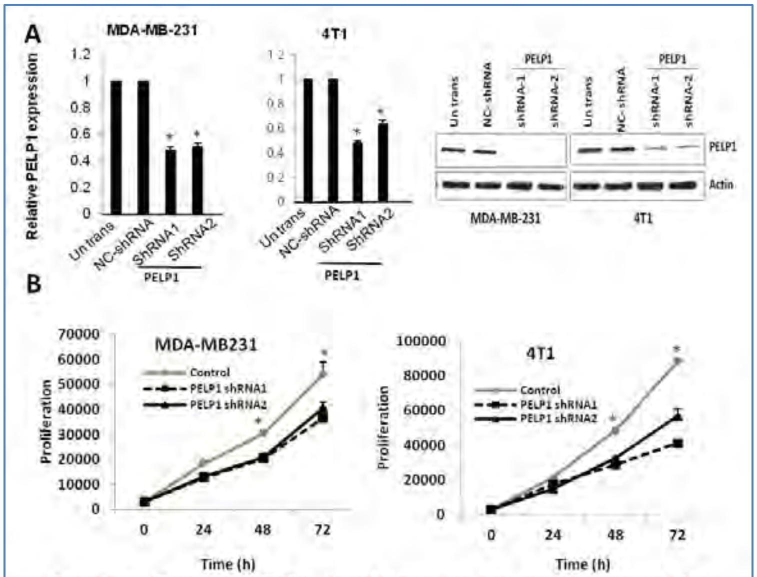

Figure 4. Effect of PELP1 knockdown on cell proliferation of ER-negative breast cancer cells. A, MDA-MB231 and 4T1 cells untransfected, and or stably expressing negative control (NC) shRNA vector or PELP1-shRNA were lysed and the expression of PELP1 was analyzed by real-time PCR analysis and Western blotting . B, Cell proliferation potential of the MDA-MB231 and 4T1 cells -PELP1-shRNA and control shRNA clones were measured *in vitro* by using a Cell Titer-Glo assay. Mean and SDs are from 3 independent experiments. *,$p \le 0.05$ *t* test.

PELP1 down regulation affects proliferation of breast cancer cells *in vitro* using the Cell Titer-Glo assay. Both PELP1shRNA model cells showed substantially less cellular proliferation than the control shRNA-transfected cells (Fig.4B). Collectively, these results indicate that the proto-oncogene PELP1 has potential to regulate the cell proliferation of ER-negative breast epithelial cells.

PELP1 signaling axis is also needed for optimal cell migration and invasion of ER-negative breast cancer cells. PELP1 expression is deregulated in metastatic tumors (9). However, whether PELP1 plays a role in the metastasis of ER-negative cells remains unknown. To examine the significance of PELP1 in ER-negative cell metastasis, we performed *in vitro* migration assays and an invasion assays using Boyden chamber assay. In the migration assays, PELP1 knock down resulted in significantly less migration in both the MDA-MB231 and 4T1 cells than in the control vector-transfected cells (Fig. 5A). PELP1 knock down also significantly reduced the invasion potential of both the MDA-MB231 and 4T1 cells (Fig. 5B). Collectively these results suggest that PELP1 has the potential to modulate migration and invasion of ER-negative breast cancer cells.

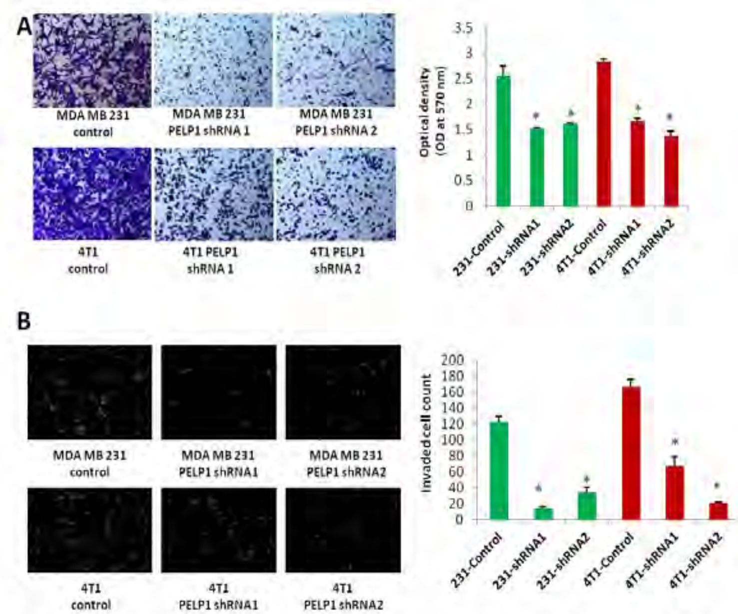

Figure 5. PELP1 knockdown affects the migration and invasion of ER-negative breast cancer cells. A, Cell migration potential of the MDA-MB231 and 4T1 cells transfected with control shRNA or PELP1-shRNA was analyzed by using a Boyden chamber assay. Photomicrographs of migrated cells in various treatments are shown. B, Cell invasion potential of the MDA-MB231 and 4T1 cells transfected with control shRNA or PELP1-shRNA was analyzed by using Matrigel invasion chamber assays. Photomicrographs of invaded cells in various treatments are shown. Mean and SDs are from 3 independent experiments. *, $p \leq 0.05$ *t* test.

KEY RESEARCH ACCOMPLISHMENTS:

Year 1

- Establishment of breast model cells model cells with functional and defective PELP1 signaling axis
- Establishment of MCF7-PELP1 and MCF7-HER2 model cells with functional and defective Src signaling axis
- Demonstration that endogenous PELP1 and Src is needed for E2 mediated ER-extranuclear signaling
- Demonstration of the significance of ER extranuclear signaling on the migratory potential of ER+ve breast cancer cells

Year 2

- Demonstration that dasatinib have therapeutic utility in blocking ER-extranuclear actions using *in vitro* models
- Demonstration that functional PELP1-Src axis is necessary for HER2 mediated ER extranuclear actions and proliferation.
- Demonstration that dasatinib have therapeutic utility in sensitizing therapy resistant cells using *in vitro* assays
- Demonstration that ER-extranuclear actions play an important role in metastases *in vivo* using xenograft models

Year 3

- Demonstration that dasatinib have therapeutic utility *in vivo* using preclinical xenograft models
- Demonstration of the significance of PELP1 siRNA nanoparticles on the proliferation of therapy resistant cells
- Demonstration of the significance of ER coregulator PELP1 signaling on the migratory potential of ER-ve breast cancer cells

REPORTABLE OUTCOMES: This study produced the following publications:

Year 1

1. Chandrasekharan Nair B and Vadlamudi RK. Regulation of hormonal therapy resistance by cell cycle machinery *Gene Therapy and Molecular Biology* 2008 Dec;12:395-404.

2. Vadlamudi RK, Rajhans R, Chakravarty D, Chandrasekharan Nair B, Nair SS, Evans DB, Chen S, Tekmal RR.. Regulation of aromatase induction by nuclear receptor coregulator PELP1. *J Steroid Biochem Mol Biol*. 118:211-218, 2010

Year 2

3. Chakravarty D, Nair SS**,** Santhanama B, Nair BC, Wang L, Bandyopadhyay A, Agyin JA, Brann D, Sun L, Yeh I, Lee FY, Tekmal R, Kumar R and Vadlamudi RK. Extranuclear functions of ER impact invasive migration and metastases of breast cancer cells. *Cancer Research*, 2010, 70(10):4092-101.

4. Chakravarty D, Tekmal R and Vadlamudi RK. PELP1: A novel therapeutic target for hormonal cancers. *IUBMB Life*. 2010 Mar;62(3):162-9.

Year 3

5. Vallabhaneni S, Nair BC, Cortez V, Challa R, Chakravarty D, Tekmal RR, Vadlamudi RK. Significance of ER-Src axis in hormonal therapy resistance. *Breast Cancer Res Treat*. 2011. [Epub ahead of print] http://www.ncbi.nlm.nih.gov/pubmed/21184269

6. Cortez V, Mann M, Brann DW, Vadlamudi RK. Extranuclear signaling by estrogen: role in breast cancer progression and metastasis. *Minerva Ginecol.* 2010 Dec;62(6):573-83.

7. Sudipa Saha Roy and Ratna K. Vadlamudi. Role of Estrogen Receptor Signaling in Breast Cancer Metastasis. *International Journal of Breast Cancer*. 2011 In press.

CONCLUSIONS:

In the first year of this study, we have generated *model* cells that have defects in PELP1-Src signaling axis. Using these models, we demonstrated that ER-extranuclear actions play an important role in cell motility, establishing for the first time that endogenous PELP1 has as a critical role in activating signaling events that lead to cell motility/invasion via ER- Src-PELP1 pathway. Our results using estrogen dendrimers (EDC) demonstrates that ER extranuclear signaling has potential to promote cytoskeleton changes, leading to increased cell migration. Our data suggest that PELP1 and Src kinase play an essential role in the activation of ER extranuclear signaling leading to cytoskeleton reorganization and migration. Since breast tumors overexpress Src kinase, deregulation of PELP1 seen in breast tumors can contribute to activation of Src kinase, leading to the progression to metastasis. Pharmacological inhibition of Src kinase using dasatinib significantly inhibited E2-mediated nongenomic actions.

In the second year of the study we found that; (a) Functional Src axis is needed for optimal activation of ERα extranuclear actions, (b) Src plays a key role in PELP1 and HER2 oncogene mediated ERα extranuclear actions and proliferation, (c) Excessive ERα extranuclear signaling in therapy resistant cells is inhibited by pharmacological inhibition of Src. Collectively, these results suggests that deregulation of PELP1 axis has the potential to contribute to breast cancer progression and therapy resistance by accelerating ER extranuclear actions. Our data using Xenograft models provided the first evidence demonstrating the significance of ER-extranuclear signaling to the metastatic potential of breast cancer cells and suggest that PELP1 deregulation commonly seen in metastastic tumors may play a role in metastasis by enhancing ER-extranuclear signaling.

In the third year of the study, we found that pharmacological inhibition of Src using dasatinib substantially inhibited the growth of therapy resistant MCF7-PELP1, MCF7-HER2, and MCF7-Tam model cells in proliferation assays. In post-menopausal xenograft based studies, treatment with dasatinib significantly inhibited the growth of therapy resistant cells. IHC analysis revealed that the tumors were ERα positive, and dasatinib treated tumors exhibited alterations in Src and MAPK signaling pathways. Combinatorial therapy of tamoxifen with dasatinib showed better therapeutic effect compared to single agent therapy on the growth of therapy resistant PELP1 driven tumors. Since PELP1, HER2, and Src kinase are commonly deregulated in breast cancers, combination therapies using both endocrine agents and dasatinib may have better therapeutic effect by delaying the development of hormonal resistance. During the third year, we also discovered that PELP1-Src pathway may play role in metastasis of ER-ve cells as well. Our ongoing studies during the fourth year (no cost extension period) will address the role of PELP1-Src axis in sensitizing ER-ve cells in vivo and finish the mechanistic studies examining the role of PELP1-Src axis in promoting metastasis.

REFERENCES:

(1) Ariazi EA, Ariazi JL, Cordera F, Jordan VC. Estrogen receptors as therapeutic targets in breast cancer. Curr Top Med Chem 2006;6:195-216.

(2) Lewis-Wambi JS, Jordan VC. Treatment of Postmenopausal Breast Cancer with Selective Estrogen Receptor Modulators (SERMs). Breast Dis 2005;24:93-105.:93-105.

(3) Leary A, Dowsett M. Combination therapy with aromatase inhibitors: the next era of breast cancer treatment? Br J Cancer 2006;.

(4) Acconcia F, Barnes CJ, Kumar R. Estrogen and tamoxifen induce cytoskeletal remodeling and migration in endometrial cancer cells. Endocrinology 2006;147:1203-12.

(5) Hall JM, McDonnell DP. Coregulators in nuclear estrogen receptor action: from concept to therapeutic targeting. Mol Interv 2005;5:343-57.

(6) Smith CL, O'Malley BW. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. Endocr Rev 2004;25:45-71.

(7) Vadlamudi RK, Balasenthil S, Sahin AA, Kies M, Weber RS, Kumar R, et al. Novel estrogen receptor coactivator PELP1/MNAR gene and ERbeta expression in salivary duct adenocarcinoma: potential therapeutic targets. Hum Pathol 2005;36:670-5.

(8) Vadlamudi RK, Kumar R. Functional and biological properties of the nuclear receptor coregulator PELP1/MNAR. Nucl Recept Signal 2007;5:e004.

(9) Rajhans R, Nair S, Holden AH, Kumar R, Tekmal RR, Vadlamudi RK. Oncogenic Potential of the Nuclear Receptor Coregulator Proline-, Glutamic Acid-, Leucine-Rich Protein 1/Modulator of the Nongenomic Actions of the Estrogen Receptor. Cancer Res 2007;67:5505-12.

(10) Rajhans R, Nair HB, Nair SS, Cortez V, Ikuko K, Kirma NB, et al. Modulation of in situ Estrogen Synthesis by PELP1: Potential ER Autocrine Signaling Loop in Breast Cancer Cells. Mol Endocrinol 2008;22:649-64.

(11) Kumar R, Zhang H, Holm C, Vadlamudi RK, Landberg G, Rayala SK. Extranuclear coactivator signaling confers insensitivity to tamoxifen. Clin Cancer Res 2009;15:4123-30.

(12) Vadlamudi RK, Manavathi B, Balasenthil S, Nair SS, Yang Z, Sahin AA, et al. Functional implications of altered subcellular localization of PELP1 in breast cancer cells. Cancer Res 2005;65:7724-32.

(13) Aslakson CJ, Miller FR. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res 1992;52:1399-405.

(14) Tavazoie SF, Alarcon C, Oskarsson T, Padua D, Wang Q, Bos PD, et al. Endogenous human microRNAs that suppress breast cancer metastasis. Nature 2008;451:147-52.

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PRECLINICAL STUDY

Significance of ER–Src axis in hormonal therapy resistance

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Abstract The estrogen receptor (ER) is implicated in the progression of breast cancer. Despite positive effects of hormonal therapy, initial or acquired resistance to endocrine therapies frequently occurs. Recent studies suggested ERα-coregulator PELP1 and growth factor receptor ErbB2/HER2 play an essential role in hormonal therapy responsiveness. Src axis couples ERα with HER2 and PELP1, thus representing a new pathway for targeted therapy resistance. To establish the significance of ER–Src axis in PELP1 and HER2 mediated therapy resistance, we have generated model cells that stably express Src-shRNA under conditions of PELP1, HER2 deregulation. Depletion of Src using shRNA substantially reduced E2 mediated activation of Src and MAPK activation in resistant model cells. Pharmacological inhibition of Src using dasatinib, an orally available inhibitor substantially inhibited the growth of therapy resistant MCF7–PELP1, MCF7–HER2, and MCF7–Tam model cells in proliferation assays. In post-menopausal xenograft based studies, treatment with dasatinib significantly inhibited the growth of therapy resistant cells. IHC analysis revealed that the tumors were ERα positive, and dasatinib treated tumors exhibited alterations in Src and MAPK signaling pathways. Combinatorial therapy of tamoxifen with dasatinib showed better therapeutic effect compared to single agent therapy on the growth of therapy resistant PELP1 driven tumors. The results from our study showed that ER–Src axis play an important role in promoting hormonal resistance by proto-oncogenes such as HER2, PELP1, and blocking this axis prevents the development of hormonal independence in vivo. Since PELP1, HER2, and Src kinase are commonly deregulated in breast cancers, combination therapies using both endocrine agents and dasatinib may have better therapeutic effect by delaying the development of hormonal resistance.

Keywords Therapy resistance · Estrogen receptor · HER2 · ER · Src · PELP1 · Breast cancer · Extranuclear signaling

Introduction

The estrogen receptor (ER) is implicated in the progression of breast cancer [1]. Endocrine therapy using tamoxifen, a selective estrogen receptor modulator (SERM) improves relapse-free and overall survival [2]. More recently aromatase inhibitors, which deplete peripheral estrogen (E2) synthesis, are shown to substantially improve disease-free survival in postmenopausal women [3]. Endocrine therapy also has a positive effect on the treatment of advanced metastatic disease. Despite these positive effects, initial or acquired resistance to endocrine therapies frequently occurs [4]. Although the mechanisms for hormonal therapy resistance remains elusive, and emerging evidence implicates human epidermal growth factor receptor-2 (HER2/ErbB2), mitogen-activated protein kinase (MAPK), and protein kinase B (AKT) pathways in development of therapy resistance [5, 6].

In addition to its well-studied nuclear functions, ER also participates in extranuclear signaling events in the cytoplasm and membrane [7]. Such signaling has been linked to

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rapid responses to estrogen which generally involves the stimulation of the Src kinase, MAPK, and AKT [8, 9]. Accumulating evidence suggests that ERα coregulators play an essential role in hormonal responsiveness and cancer progression [8, 10, 11]. Proline, glutamic-acid, and leucine-rich protein 1 (PELP1) is an ERα-coregulator that functions in nuclear as well as in extranuclear actions [12]. PELP1, a recently discovered proto-oncogene [13], exhibits aberrant expression in many hormone-related cancers [14] and is a prognostic indicator of shorter breast cancer-specific survival and disease-free intervals when over-expressed [15].

Proto-oncogene c-Src is a multifunctional intracellular tyrosine kinase implicated in the regulation of a variety of processes including proliferation, differentiation, survival, and motility [16]. Src interacts with multiple cellular factors including HER2, ERα, PELP1 and breast tumors frequently over-express Src kinase [17]. Dasatinib (Trade name SPRYCEL), an orally available inhibitor of Src family tyrosine kinases that is currently approved by FDA for leukemia, is now in phase I and II clinical trials for treatment of solid tumors [18, 19]. Recent evidence suggest that ERα-coregulator PELP1 plays an essential role in coupling ERα with Src kinases leading to hormonal resistance and that PELP1–Src interactions play an important role in PELP1 functions [14].

In this study, we examined whether ERα–Src axis constitutes a critical pathway used by breast cancer cells for developing resistance. Using Src-shRNA, Src inhibitor dasatinib and therapy resistant model cells, we demonstrate that a functional Src axis is essential for ERα-coregulator mediated extranuclear actions. Our results also suggest that ERα–PELP1 coregulator-Src axis plays an important role in promoting hormonal resistance by oncogenes and blocking this axis reduces growth of therapy resistant cells in vivo and that dasatinib may have a therapeutic potential of treating hormonal therapy resistance.

Materials and methods

Reagents

MCF7 cells were purchased from American-type culture collection (ATCC, Manassas, VA). 17*β* estradiol, tamoxifen, and Actin antibody were purchased from Sigma Chemical Co (St. Louis, MO). PELP1 antibody was purchased from Bethyl laboratories (Montgomery, TX). Antibodies against phospho-AKT and total AKT, phospho-MAPK and total MAPK, phospho-Src and total c-Src were purchased from Cell Signaling (Beverly, MA). Dasatinib was obtained from LC Laboratories (Woburn, MA).

Model cells

MCF7–PELP1 cells [20], MCF7–HER2 [21], and MCF7–Tam cells [21] were earlier described. MCF7–PELP1 and MCF7–HER2 cells stably expressing Src-shRNA were generated using validated Src-shRNA lentivitral particles (SHCLMV) purchased from Sigma and using Puromycin selection (1 μg/ml).

Western blotting

Model cells were cultured in RPMI Media containing 5% Dextran Charcoal treated Serum for at least 48 h prior to estrogen treatment (100 nM). Cells were washed with phosphate buffer saline (PBS) after 5 min of treatment and lysis was done using RIPA buffer containing phosphatase and protease inhibitors and samples were run on either 7% or 10% SDS-PAGE. Western blot analysis with either phospho antibodies or total antibodies was performed as previously described [22].

Cell proliferation assay

Cell proliferation rate was measured using a 96-well format with Cell Titer-Glo Luminescent Cell Viability Assay (Promega; G7572). 5×10^3 cells were plated in each well of a Corning® 96 well flat clear bottom, opaque wall microplates and cultured in RPMI Media containing 2.5% DCC treated serum for 24 h and followed by treatment with or without estrogen (100 nM/well) for another 72 h. Luminescence was recorded using automatic Fluoroskan Luminometer as per the manufacturer's recommendation.

In vivo tumorigenesis assays

For tumorigenesis studies, model cells (5×10^6 cells) were implanted subcutaneously into the flanks of 6- to 7-week-old female nude mice as described [23]. Nude mice (nu/nu) were injected with control MCF7 cells or MCF7 cells that over-express PELP1 by mixing them with equal volume of Matrigel. Because athymic mice were deficient in adrenal androgens, they were supplemented with sub-cutaneous injections of the aromatase substrate androstenedione (100 μg/day) for the duration of the experiment as described for the postmenopausal model [24]. Treatment was initiated after 3 weeks of inoculation and treatment with dasatinib (15 μg/mouse/day/oral), or combination with tamoxifen (100 μg/mouse/day/subcutaneous) or letrozole (15 μg/mouse/day) was continued for 6 weeks. Tumor volumes were measured with a vernier caliper at weekly intervals. After 6 weeks, mice were euthanized, and tumors were removed, weighed and processed for IHC staining. Tumor volume was calculated using a modified ellipsoidal

formula: tumor volume = $1/2(L \times W^2)$, where L is the longitudinal diameter and W is the transverse diameter [25, 26].

Immunohistochemistry

Immunohistochemical analysis was performed using a method as described [27]. PELP1 antibody (IHC-00013, 1:750) from Bethyl Lab, ERα (SC-7207; 1:50), and phosphor-Tyr419-c-Src (sc-101802; 1:50) from Santa Cruz Biotech, Inc, phospho-MAPK (4376s; 1:100) from Cell Signaling, PCNA (VP-P980; 1:100) from Vector Lab were used in conjunction with proper controls, visualized by DAB substrate (Vector Lab) and counterstained with hematoxylin (Vector Lab, Inc. CA). TUNEL analysis was done using the In situ Cell Death Detection Kit (Roche; 11684 795910) as per the manufacturer's protocol. Apoptotic cells were identified by positive TUNEL staining and five randomly selected microscopic fields in each group were used to calculate the relative ratio of TUNEL-positive cells.

Statistical analysis

Statistical differences among groups were analyzed with either t test or ANOVA as appropriate using SPSS software.

Results

Functional Src kinase axis is needed for PELP1 dependent ER actions

Recent studies established PELP1 as an independent prognostic indicator of shorter breast cancer-specific survival and disease-free intervals [15]. In earlier studies, we have established breast cancer model cells with stable expression of PELP1 (MCF7–PELP1) to mimic the situation commonly seen in a subset of breast tumors. These model cells express two- to threefold more expression of PELP1 compared to endogenous levels of PELP1 and exhibit increased E2 mediated proliferation [28], therapy resistance [29], and tumorigenesis in xenograft models [13]. To study the in vivo significance of Src kinase in PELP1 mediated actions, we established MCF7–PELP1 model cells (pooled clones) stably expressing Src-shRNA using a lentivirus system with puromycin selection. Western blot analysis of total lysates from PELP1–Src-shRNA clones revealed that the Src-shRNA down regulated Src expression to ~75% of the level seen in the parental MCF7–PELP1 and the vector–transfected control clones (Fig. 1a). Src knockdown did not affect the expression of ERα in these clones (Fig. 1b). Since PELP1 participates in ERα-extranuclear actions, we examined the significance of endogenous Src in the activation of ERα-extranuclear signaling pathways. We measured the activation of signaling pathways including Src, and MAPK after treating cells with estrogen (E2) for 5 min. Estrogen addition uniquely promoted activation of Src and MAPK pathways in MCF7 cells. As observed before, MCF7–PELP1 cells showed further increase in activation of MAPK compared to MCF7 cells. Src-shRNA-expressing MCF7–PELP1 cells had significantly less Src, and MAPK activation (Fig. 1c). We then examined whether Src down regulation affected PELP1-mediated increase in E2 driven proliferation using a Cell Titer-Glo assay. PELP1 expression increased estrogen-mediated cellular proliferation compared to MCF7 cells, while Src downregulation in MCF7–PELP1 clones diminished its ability to increase cell proliferation (Fig. 1d).

HER2-mediated ER extranuclear actions requires Src kinase

Deregulation of HER2 expression/signaling has emerged as the most significant factor in the development of hormonal resistance [9, 20] and cross-talk between the ER and HER2 pathways has been shown to promotes endocrine therapy resistance [20, 21]. ERα-coregulator PELP1 interacts with HER2 and is implicated in facilitating the ERα crosstalk with HER2 signaling pathways [29]. To examine whether Src axis plays a role in HER2 mediated ERα extranuclear actions, we have down regulated Src kinase using shRNA delivery. We have established two pooled clones of Src-shRNA in a MCF7–HER2 background, a well-established model cell for HER2 deregulation [21]. Western blot analysis of HER2–Src-shRNA clones revealed 85–90% decrease in Src expression compared to the level seen in the parental MCF7–HER2–vector clone (Fig. 2a). Src knockdown did not significantly affect the expression of ERα in these clones (Fig. 2b). To examine the significance of endogenous Src in the HER2 mediated activation of ERα-extranuclear signaling, we measured the activation of Src, and MAPK after treating cells with estrogen for 5 min. Estrogen addition uniquely promoted activation of Src and MAPK pathways in MCF7 cells and MCF7–HER2 cells showed excessive activation of MAPK and AKT pathways. However, Src-shRNA-expressing MCF7–HER2 cells had significantly less AKT and MAPK activation (Fig. 2c). In cell proliferation assays, MCF7–HER2 cells showed significantly increased proliferation compared MCF7 cells, while Src-shRNA-expressing MCF7–HER2 clones showed decreased proliferation compared to parental MCF7–HER2 cells (Fig. 2d). Collectively, these results suggest that functional Src axis is necessary for HER2 mediated ER extranuclear actions and proliferation.

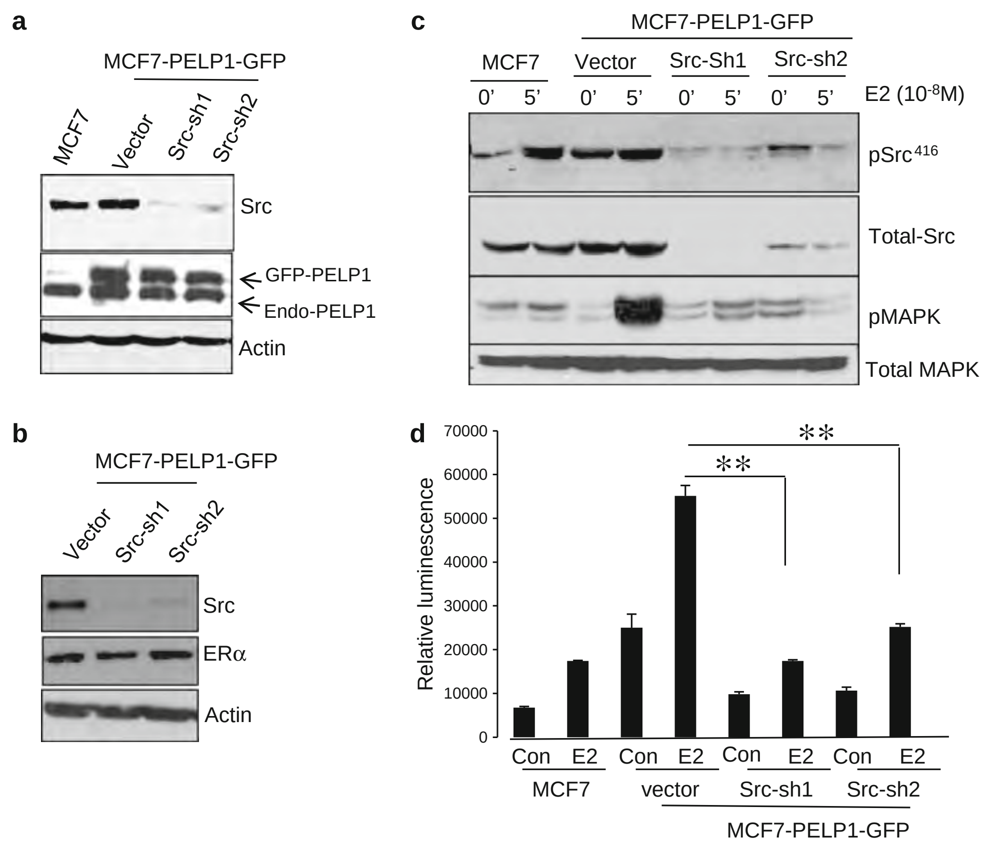

Fig. 1 Down regulation of Src kinase reduces PELP1 mediated ER extra-nuclear signaling. **a** MCF7–shRNA, MCF7–PELP1, and MCF7–PELP1–Src-shRNA cells were lysed and expression of Src was analyzed by western blotting. **b** Total lysates from MCF7–PELP1 and MCF7–PELP1–Src-shRNA model cells were analyzed for the expression of ERα by western blotting. **c** MCF7, MCF7–PELP1, and MCF7–PELP1–Src-shRNA cells were cultured in 5% DCC serum containing medium treated with or without estrogen. Activation of Src and MAPK signaling pathways was analyzed by western blotting of total protein lysates with phospho-specific antibodies. **d** Cell proliferation capacity of MCF7, MCF7–PELP1, and MCF7–PELP1–Src-shRNA stable cells were analyzed after treating the cells with or without E2 using Cell Titer-Glo assay. $^{**}P < 0.001$

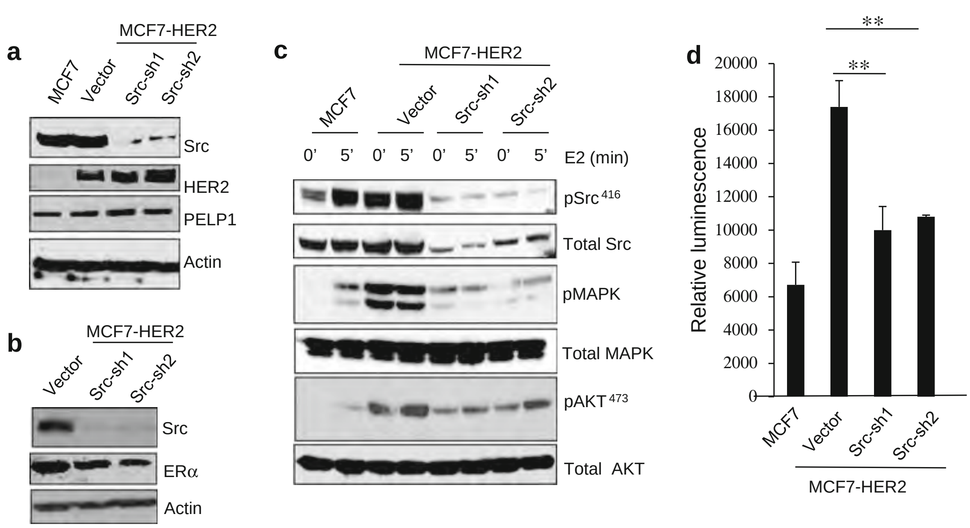

Fig. 2 Down regulation of Src kinase reduces HER2 mediated ER extra-nuclear signaling. **a** MCF7–shRNA, MCF7–HER2, and MCF7–HER2–Src-shRNA cells were lysed and expression of Src was analyzed by western blotting. **b** Total lysates from MCF7–shRNA, MCF7–HER2, and MCF7–HER2–Src-shRNA were analyzed for the expression of ERα, by western blotting. **c** MCF7–shRNA, MCF7–HER2, and MCF7–HER2–Src-shRNA cells were cultured in 5% DCC serum containing medium treated with or without E2. The activation of Src, AKT, and MAPK signaling pathways was analyzed by western blotting of total protein lysates with phospho-specific antibodies. **d** Cell proliferation capacity of MCF7–shRNA, MCF7–HER2, and MCF7–HER2–Src-shRNA stable cells were analyzed using Cell Titer-Glo assay. $^{**}P < 0.001$

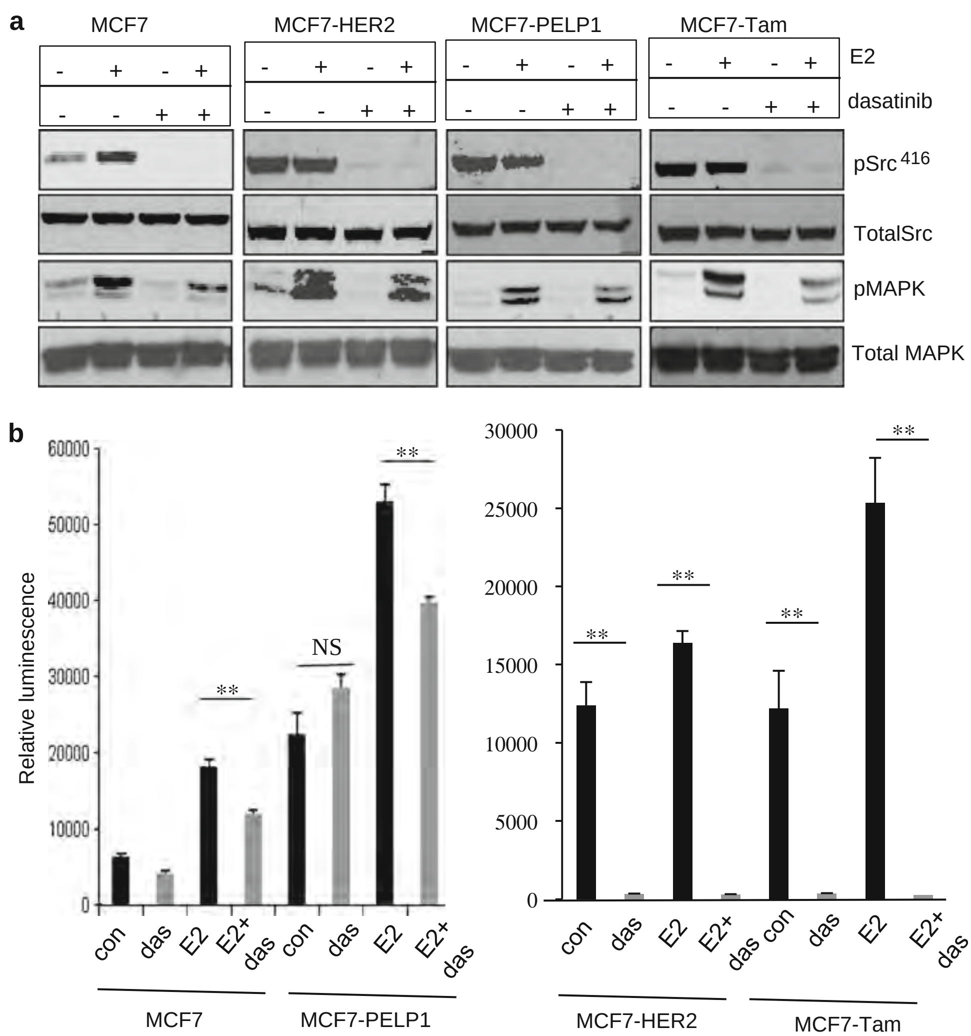

Fig. 3 Dasatinib reduces E2 mediated ER extranuclear actions on therapy resistant cells. **a** MCF7, MCF7–HER2, MCF7–PELP1, and MCF7–Tam cells were cultured in 5% DCC serum containing medium treated with or without E2 in the presence or absence of dasatinib. The activation of Src, and MAPK signaling pathways was analyzed by western blotting of total protein lysates with phospho-specific antibodies. **b** Cell proliferation capacity of MCF7 and MCF7–PELP1 cells were analyzed after treating the cells with or without E2 in the presence or absence of dasatinib using Cell Titer-Glo assay. **c** Cell proliferation capacity of MCF7–Tam, and MCF7–HER2 cells were analyzed after treating the cells with or without dasatinib using Cell Titer-Glo assay. *$P < 0.05$; **$P < 0.001$

Dasatinib blocks estrogen-mediated ER extranuclear actions in therapy resistant cells

Earlier studies have shown that MCF7–PELP1 and MCF7–HER2 model cells exhibit hormonal therapy resistance. Since our findings suggested that Src kinase plays a key role in estrogen-mediated extranuclear signaling in these cells, we examined the effect of pharmacological inhibition of Src kinase using dasatinib, a well-established orally available inhibitor of Src family tyrosine kinases [19]. In addition to inhibiting SRC in the subnanomolar range, dasatinib also variably inhibits other SFKs, c-KIT, PDGFR, and ephrin A2 [30]. As a second model, we have used MCF7–Tam model cells, a well-studied model cells that exhibit acquired resistance to tamoxifen [21]. Short time estrogen treatment of MCF7 cells resulted in increased activation Src and MAPK pathways (Fig. 3a). Interestingly, all three resistant model cells have constitutively higher levels of Src activation and estrogen treatment substantially increased activation of MAPK in these model cells compared to therapy sensitive MCF7 cells (Fig. 3a). Dasatinib pretreatment abolished estrogen-mediated activation of Src and MAPK pathways in therapy sensitive MCF7 and also in all three therapy resistant models (Fig. 3a). In estrogen driven proliferation assays, dasatinib (200 nM) treatment substantially reduced PELP1 mediated increase in estrogen driven cell proliferation (Fig. 3b). Similarly, dasatinib (200 nM) treatment also reduced the proliferation of therapy resistant MCF–Tam and MCF7–HER2 cells. Collectively, these results suggest that Src signaling plays a role in proliferation of therapy resistant cells and dasatinib can potentially be used to reduce estrogen-mediated extranuclear signaling in therapy resistant cells.

Dasatinib decreases PELP1 oncogenic potential in vivo

PELP1 deregulation promotes in vivo tumorigensis [13]. PELP1 also promotes local estrogen synthesis via Src kinase pathway facilitating growth of tumors in an autocrine

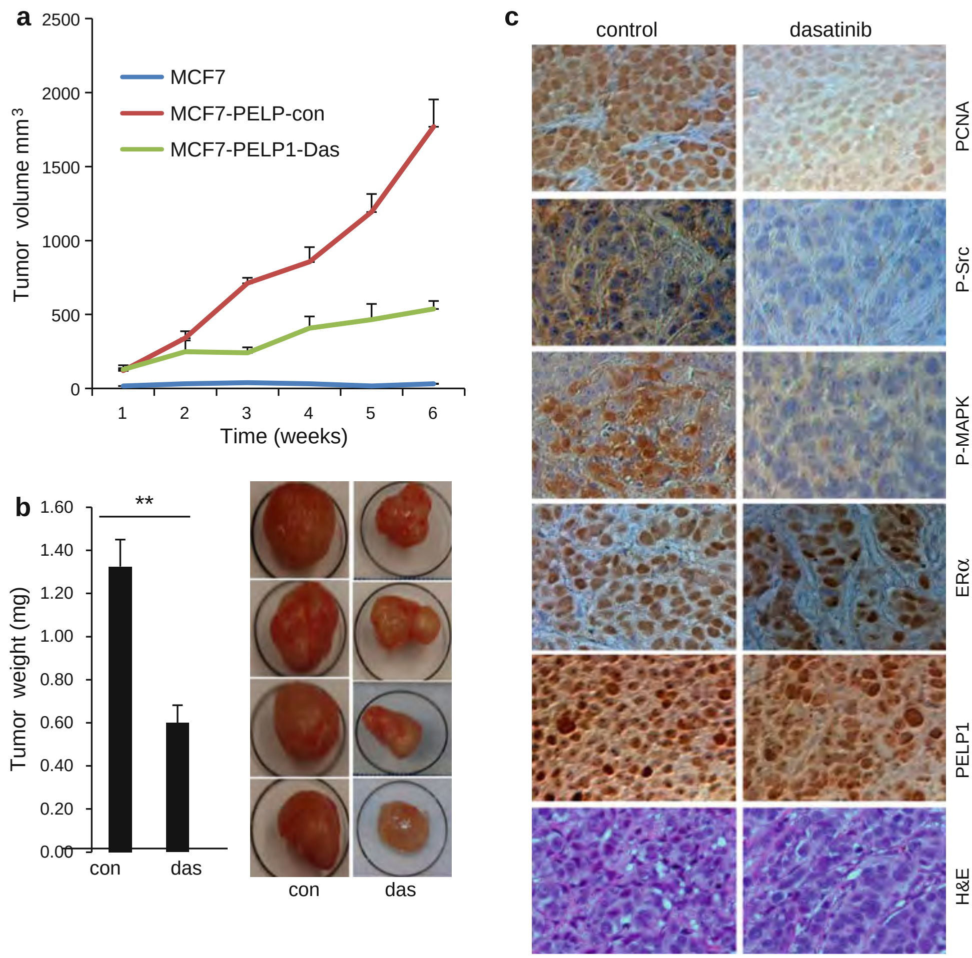

Fig. 4 Dasatinib reduces PELP1 driven cancer progression in vivo. **a** Nude mice were injected subcutaneously with MCF7 or MCF7–PELP1–WT. When the tumor reached ~100 mm^3, it was treated with or without dasatinib ($n = 4$). Tumor growth was measured at weekly intervals. Tumor volume is shown in the graph. **b** The average tumor weight is shown in the graph. Representative images of tumors are shown. **c** Status of PELP1, ER, Src phosphorylation and PCNA expression as a marker of proliferation was analyzed by immunohistochemistry (IHC). *$P < 0.05$

manner [31]. Therefore, we hypothesized that PELP1 driven tumors can be therapeutically targeted using dasatinib and performed a proof of principle experiment using a postmenopausal xenograft model. Nude mice (nu/nu) were injected with control MCF7 cells or MCF7–PELP1 cells that overexpress PELP1 by mixing them with equal volume of Matrigel. Because athymic mice were deficient in adrenal androgens, they were supplemented daily with s.c. injections of the aromatase substrate androstenedione (100 µg/day) for the duration of the experiment. Under these conditions, injected MCF7 cells did not form tumors. As observed before, MCF7–PELP1 expressing cells formed tumors in the absence of exogenous estrogen supplementation suggesting local derived estrogen supported the growth of MCF7–PELP1 cells. When the tumor volume reached 100 mm^3, mice ($n = 4$) were either treated with dasatinib (15 mg/kg/day/oral) or treated with vehicle (citrate buffer). Dasatinib treatment significantly reduced the PELP1-driven tumor volume (Fig. 4a) and tumor weight (Fig. 4b). Dasatinib treated tumors revealed decreased proliferation as evidenced by decreased nuclear PCNA staining and exhibited decreased Src and MAPK kinase activity seen by diminished phospho antibody staining (Fig. 4c). These results suggested that functional Src–MAPK axis is needed for PELP1 mediated tumorigenesis in vivo and dasatinib can be potentially used to reduce PELP1 mediated tumor growth.

Dasatinib in combination with antiestrogens reduces PELP1 mediated therapy resistance

PELP1 deregulation is known to promote tamoxifen resistance [29, 32]. We therefore examined whether dasatinib reduces PELP1-mediated therapy resistance. Xenografts established as described in earlier section were randomly assigned to groups ($n = 4$) and treated with tamoxifen, letrozole, dasatinib alone or in combination. As seen in previously published studies [32], tamoxifen did not affect the growth of PELP1 driven tumors, while dasatinib substantially inhibited tumor volume (Fig. 5a) and weight (Fig. 5b). Combination treatment of tamoxifen with dasatinib showed further decreases in tumor volume and growth compared dasatinib alone, however, the differences are not statistically significant. Similarly, letrozole, an aromatase inhibitor, substantially reduced PELP1 driven tumor growth

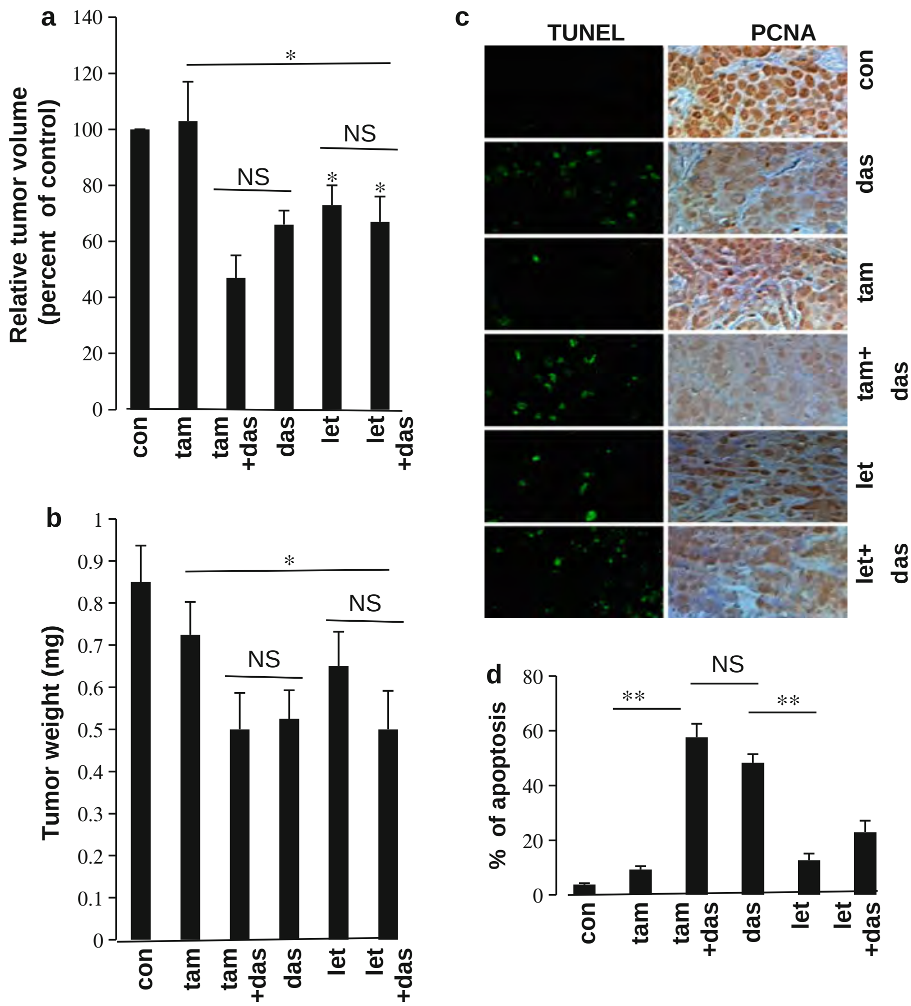

Fig. 5 Antitumor effects of dasatinib in combination with tamoxifen and letrozole. **a** Nude mice were injected subcutaneously with MCF7–PELP1–WT. When the tumor volume reached ~100 mm^3, then dasatinb or combination treatment was started ($n = 4$) and tumor growth was measured at weekly intervals. Tumor volume is shown in the graph. **b** The average tumor weight is shown in the graph. **c** TUNEL staining as a marker of apoptosis and PCNA expression as a marker of proliferation was analyzed by immunohistochemistry (IHC). *$P < 0.05$. **d** Representative results and quantitative analysis of TUNEL staining. Columns, mean TUNEL labeling percentage based on five randomly selected high-power microscopic fields for each group. **$P < 0.001$. *NS* non significant

underscoring the importance of locally synthesized estrogen in PELP1 driven tumor growth, however, the combination of letrozole with dasatinb only slightly enhanced the therapy response. IHC examination of PCNA staining revealed that dasatinib treatment decreased proliferation of tumor cells, while TUNEL staining showed increased apoptosis in dasatinb treated tumor cells. Collectively, these results suggest that pharmacological inhibition of Src could be used to treat therapy resistance induced by deregulation of proto-oncogene PELP1.

Discussion

Estradiol (E2), ER, and ER coregulators have been implicated in the development and progression of breast cancer. Two thirds of breast tumors express ERα and women having ER+ve tumors are treated with endocrine therapy and tumors appear to use adaptive mechanisms for growth after the initiation of first-line endocrine therapy and hormonal therapy resistance is a major clinical problem [33]. In this study, we found that; (a) functional Src axis is needed for optimal activation of ERα extranuclear actions, (b) Src plays a key role in PELP1 and HER2 oncogene mediated ERα extranuclear actions and proliferation, (c) Excessive ERα extranuclear signaling in therapy resistant cells is inhibited by pharmacological inhibition of Src, (d) Functional Src axis is needed for PELP1 mediated oncogenic functions in vivo, and (e) Combination therapy of Src inhibitor with antiestrogens is more effective in blocking therapy resistance. Collectively, these results suggests that deregulation of PELP1 axis has the potential to contribute to breast cancer progression and therapy resistance by accelerating ER extranuclear actions.

It is increasingly clear that ERα also participates in cytoplasmic and membrane-mediated signaling events (extragenomic signaling) and generally involves cytosolic kinases including Src, MAPK, PI3K [7, 34]. Accumulating evidence strongly suggests that ER signaling requires coregulatory proteins and their composition in a given cell determine the magnitude and specificity of the ERα signaling [35, 36]. Some evidence suggests that the extranuclear

effects of estrogen can regulate different cellular processes, such as proliferation, survival, and apoptosis. However, the pathological significance of ER extranuclear signaling and its role in therapy resistance remain unknown. Our results show that therapy resistant cells exhibit excessive activation of ERα extranuclear actions and blockage of endogenous Src axis either by Src specific shRNA or Src inhibitors significantly attenuated ER extranuclear actions and resulted in reduced proliferation. Collectively, our results suggest that ERα extranuclear action involves Src kinase and deregulation of Src kinase seen in breast tumors may have implications for potential activation of ERα extranuclear actions leading to therapy resistance.

PELP1, a recently discovered proto-oncogene [13], exhibits aberrant expression in many hormone-related cancers [14] and over-expression of PELP1 is a prognostic indicator of shorter breast cancer-specific survival and disease-free intervals [15]. In our studies, we found that PELP1-driven tumors are ERα positive and have excessive activation of Src and MAPK pathways. Inhibition of Src pathway using the orally available Src kinase inhibitor dasatinib substantially reduced PELP1-driven tumor growth with concurrent reduction in the activation of Src and MAPK pathways. These results further implicate that PELP1–Src axis mediated ER extranuclear actions may play role in breast tumorigenesis.

Src interacts with multiple cellular factors including HER2, EGFR, ERα, and breast tumors over-express Src kinase [17]. Emerging evidence suggests that PELP1 acts as a scaffolding protein coupling ER with Src kinase leading to activation of ERα–Src–MAPK pathway [14]. Mutational analysis of ERα and c-Src mutants revealed that PELP1 interacts with c-Src SH3 domain via its N-terminal PXXP motif. ERα interacts with Src's SH2 domain at phosphotyrosine 537, and the PELP1–ER interaction further stabilizes this complex [6]. Since breast tumors over-express wild type Src kinase, deregulation of PELP1 seen in breast tumors can contribute to activation of Src kinase leading to excessive activation of ER–PELP1–Src signaling pathway.

HER2, an oncogene that is overexpressed, amplified, or both, in several human malignancies including breast tumors. ERα expression occurs in $\sim$50% HER2 positive breast cancers and cross-talk between the ER and HER2 pathways promotes endocrine therapy resistance [37, 38]. ERα coregulators are also targeted by excessive ERα–HER2 crosstalk leading to hormonal resistance in a subset of breast tumors [39]. Further, HER2 overexpression can also promote ligand-independent recruitment of coactivator complexes to E2-responsive promoters and thus may play a role in the development of therapy resistance [40]. ERα-coregulator PELP1 interacts with HER2, and growth factor signaling to promote phosphorylation of PELP1 [29]. Deregulation of HER2 signaling is known to modulate PELP1 function leading to enhanced aromatase activation via Src kinase [31]. Our study suggests that endogenous Src plays an important role in HER2–ER crosstalk leading to activation of MAPK and AKT pathways.

While hormonal resistance can occur via multiple mechanisms, understanding the key pathways for resistance and targeting them is essential to extend or restore sensitivity of the therapeutic effect of tamoxifen or other SERMs. Since multiple signaling pathways in addition to hormones are involved in activating ERα [6, 37], combination therapies using both endocrine and non-endocrine agents that block different pathways may have a better therapeutic effect and may delay development of hormonal resistance. Our results suggest that the Src inhibitor dasatinib, represents a non-endocrine drug that could be used to block ERα extranuclear actions. Dasatinib efficiently blocked the activation of ERα-mediated extranuclear signals in three different models of therapy resistance. Further, dasatinib also inhibited ER-coregulator mediated tumorogenesis in vivo and dasatinib treatment sensitized PELP1 driven tumor to tamoxifen therapy. These findings suggest that dasatinib has the potential to block ERα–PELP1-mediated extranuclear signals and thus may serve as an alternative to non-endocrine drug for combinatorial therapy.

In summary, our data provide evidence demonstrating the significance of ER–PELP1–Src axis mediated extranuclear signaling to the therapy resistance. Our findings also identified Src as a novel therapeutic target for blocking of ERα–PELP1 signals and the Src inhibitor dasatinib represents a novel drug to prevent the emergence of therapy resistance in combination with endocrine therapy.

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Conflicts of interest None.

References

1. Ariazi EA, Ariazi JL, Cordera F, Jordan VC (2006) Estrogen receptors as therapeutic targets in breast cancer. Curr Top Med Chem 6:195–216
2. Lewis-Wambi JS, Jordan VC (2005) Treatment of postmenopausal breast cancer with selective estrogen receptor modulators (SERMs). Breast Dis 24:93–105
3. Leary A, Dowsett M (2006) Combination therapy with aromatase inhibitors: the next era of breast cancer treatment? Br J Cancer 95:661–666
4. Musgrove EA, Sutherland RL (2009) Biological determinants of endocrine resistance in breast cancer. Nat Rev Cancer 9:631–643
5. Gururaj AE, Rayala SK, Vadlamudi RK, Kumar R (2006) Novel mechanisms of resistance to endocrine therapy: genomic and nongenomic considerations. Clin Cancer Res 12:1001s–1007s

6. Schiff R, Massarweh SA, Shou J, Bharwani L, Arpino G, Rimawi M, Osborne CK (2005) Advanced concepts in estrogen receptor biology and breast cancer endocrine resistance: implicated role of growth factor signaling and estrogen receptor coregulators. Cancer Chemother Pharmacol 56(Suppl 1):10–20
7. Losel R, Wehling M (2003) Nongenomic actions of steroid hormones. Nat Rev Mol Cell Biol 4:46–56
8. Acconcia F, Kumar R (2005) Signaling regulation of genomic and nongenomic functions of estrogen receptors. Cancer Lett 238:1–14
9. Song RX, Zhang Z, Santen RJ (2005) Estrogen rapid action via protein complex formation involving ERalpha and Src. Trends Endocrinol Metab 16:347–353
10. Hall JM, McDonnell DP (2005) Coregulators in nuclear estrogen receptor action: from concept to therapeutic targeting. Mol Interv 5:343–357
11. Smith CL, O'Malley BW (2004) Coregulator function: a key to understanding tissue specificity of selective receptor modulators. Endocr Rev 25:45–71
12. Vadlamudi RK, Wang RA, Mazumdar A, Kim Y, Shin J, Sahin A, Kumar R (2001) Molecular cloning and characterization of PELP1, a novel human coregulator of estrogen receptor alpha. J Biol Chem 276:38272–38279
13. Rajhans R, Nair S, Holden AH, Kumar R, Tekmal RR, Vadlamudi RK (2007) Oncogenic potential of the nuclear receptor coregulator proline-, glutamic acid-, leucine-rich protein 1/modulator of the nongenomic actions of the estrogen receptor. Cancer Res 67:5505–5512
14. Vadlamudi RK, Kumar R (2007) Functional and biological properties of the nuclear receptor coregulator PELP1/MNAR. Nucl Recept Signal 5:e004
15. Habashy HO, Powe DG, Rakha EA, Ball G, Macmillan RD, Green AR, Ellis IO (2009) The prognostic significance of PELP1 expression in invasive breast cancer with emphasis on the ER-positive luminal-like subtype. Breast Cancer Res Treat 120: 603–612
16. Trevino JG, Summy JM, Gallick GE (2006) SRC inhibitors as potential therapeutic agents for human cancers. Mini Rev Med Chem 6:681–687
17. Russello SV, Shore SK (2004) SRC in human carcinogenesis. Front Biosci 9:139–144
18. Huang F, Reeves K, Han X, Fairchild C, Platero S, Wong TW, Lee F, Shaw P, Clark E (2007) Identification of candidate molecular markers predicting sensitivity in solid tumors to dasatinib: rationale for patient selection. Cancer Res 67:2226–2238
19. Summy JM, Gallick GE (2006) Treatment for advanced tumors: SRC reclaims center stage. Clin Cancer Res 12:1398–1401
20. Nagpal JK, Nair S, Chakravarty D, Rajhans R, Pothana S, Brann DW, Tekmal RR, Vadlamudi RK (2008) Growth factor regulation of estrogen receptor coregulator PELP1 functions via protein kinase A pathway. Mol Cancer Res 6:851–861
21. Nabha SM, Glaros S, Hong M, Lykkesfeldt AE, Schiff R, Osborne K, Reddy KB (2005) Upregulation of PKC-delta contributes to antiestrogen resistance in mammary tumor cells. Oncogene 24:3166–3176
22. Dimple C, Nair SS, Rajhans R, Pitcheswara PR, Liu J, Balasenthil S, Le XF, Burow ME, Auersperg N, Tekmal RR, Broaddus RR, Vadlamudi RK (2008) Role of PELP1/MNAR signaling in ovarian tumorigenesis. Cancer Res 68:4902–4909
23. Vadlamudi RK, Bagheri-Yarmand R, Yang Z, Balasenthil S, Nguyen D, Sahin AA, den Hollanden P, Kumar R (2004) Dynein light chain 1, a p21-activated kinase 1-interacting substrate, promotes cancerous phenotypes. Cancer Cell 5:575–585
24. Long BJ, Jelovac D, Handratta V, Thiantanawat A, MacPherson N, Ragaz J, Goloubeva OG, Brodie AM (2004) Therapeutic strategies using the aromatase inhibitor letrozole and tamoxifen in a breast cancer model. J Natl Cancer Inst 96:456–465
25. Jensen MM, Jorgensen JT, Binderup T, Kjaer A (2008) Tumor volume in subcutaneous mouse xenografts measured by microCT is more accurate and reproducible than determined by 18F-FDG-microPET or external caliper. BMC Med Imaging 8:16
26. Euhus DM, Hudd C, LaRegina MC, Johnson FE (1986) Tumor measurement in the nude mouse. J Surg Oncol 31:229–234
27. Vadlamudi RK, Balasenthil S, Sahin AA, Kies M, Weber RS, Kumar R, El-Naggar AK (2005) Novel estrogen receptor coactivator PELP1/MNAR gene and ERbeta expression in salivary duct adenocarcinoma: potential therapeutic targets. Hum Pathol 36:670–675
28. Balasenthil S, Vadlamudi RK (2003) Functional interactions between the estrogen receptor coactivator PELP1/MNAR and retinoblastoma protein. J Biol Chem 278:22119–22127
29. Vadlamudi RK, Manavathi B, Balasenthil S, Nair SS, Yang Z, Sahin AA, Kumar R (2005) Functional implications of altered subcellular localization of PELP1 in breast cancer cells. Cancer Res 65:7724–7732
30. Chang Q, Jorgensen C, Pawson T, Hedley DW (2008) Effects of dasatinib on EphA2 receptor tyrosine kinase activity and downstream signalling in pancreatic cancer. Br J Cancer 99:1074–1082
31. Rajhans R, Nair HB, Nair SS, Cortez V, Ikuko K, Kirma NB, Zhou D, Holden AE, Brann DW, Chen S, Tekmal RR, Vadlamudi RK (2008) Modulation of in situ estrogen synthesis by PELP1: potential ER autocrine signaling loop in breast cancer cells. Mol Endocrinol 22:649–664
32. Kumar R, Zhang H, Holm C, Vadlamudi RK, Landberg G, Rayala SK (2009) Extranuclear coactivator signaling confers insensitivity to tamoxifen. Clin Cancer Res 15:4123–4130
33. Ali S, Coombes RC (2002) Endocrine-responsive breast cancer and strategies for combating resistance. Nat Rev Cancer 2: 101–112
34. Bjornstrom L, Sjoberg M (2005) Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. Mol Endocrinol 19:833–842
35. Lonard DM, O'Malley BW (2006) The expanding cosmos of nuclear receptor coactivators. Cell 125:411–414
36. Collingwood TN, Urnov FD, Wolffe AP (1999) Nuclear receptors: coactivators, corepressors and chromatin remodeling in the control of transcription. J Mol Endocrinol 23:255–275
37. Schiff R, Massarweh S, Shou J, Osborne CK (2003) Breast cancer endocrine resistance: how growth factor signaling and estrogen receptor coregulators modulate response. Clin Cancer Res 9: 447S–454S
38. Marcom PK, Isaacs C, Harris L, Wong ZW, Kommarreddy A, Novielli N, Mann G, Tao Y, Ellis MJ (2006) The combination of letrozole and trastuzumab as first or second-line biological therapy produces durable responses in a subset of HER2 positive and ER positive advanced breast cancers. Breast Cancer Res Treat 102:43–49
39. Shou J, Massarweh S, Osborne CK, Wakeling AE, Ali S, Weiss H, Schiff R (2004) Mechanisms of tamoxifen resistance: increased estrogen receptor–HER2/neu cross-talk in ER/HER2-positive breast cancer. J Natl Cancer Inst 96:926–935
40. Shin I, Miller T, Arteaga CL (2006) ErbB receptor signaling and therapeutic resistance to aromatase inhibitors. Clin Cancer Res 12:1008s–1012s

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Extranuclear signaling by estrogen: role in breast cancer progression and metastasis

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The estrogen receptor (ERα) is implicated in the progression of breast cancer. Hormonal therapies which block ER functions or local and systemic estrogen production are currently used to treat hormonal positive breast cancer. Hormonal therapy shows beneficial effects, however, initial or acquired resistance to endocrine therapies frequently occurs, and tumors recur as metastasis. Emerging evidence suggests in addition to exerting its well-studied nuclear functions, ERα also participates in extranuclear signaling that involve growth factor signaling components, adaptor molecules and the stimulation of cytosolic kinases. ERα extranuclear pathways have the potential to activate gene transcription, modulate cytoskeleton, and promote tumor cell proliferation, survival, and metastasis. Cytoplasmic/membrane ERα is detected in a subset of breast tumors and expression of extranuclear components ERα is deregulated in tumors. The extranuclear actions of ER are emerging as important targets for tumorigenic and metastatic control. Inhibition of ERα extranuclear actions has the potential to prevent breast tumor progression and may be useful in preventing ERα positive metastasis. In this review, we summarize the results of recent research into the role of ERα mediated extranuclear actions in breast tumorigenesis and metastasis.

Key words: Estrogens – Breast neoplasms - Neoplasm metastasis.

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Estrogens regulate the expression and activity of key signaling molecules critical in various cellular signaling pathways. The biological effects of estrogen are mediated by its binding to structurally and functionally distinct estrogen receptors, alpha and beta (ERα and ERβ).[1] ER functions as a ligand-activated transcription factor, providing a direct link between intra- and extracellular signaling molecules resulting in the regulation of numerous critical cellular processes including growth, development, differentiation and maintenance within a diverse range of mammalian tissues.

ERs consist of a N-terminal region (A/B domain) containing a constitutively active ligand-independent transactivation (AF1) domain whose activity is regulated by phosphorylation via activation of signaling kinases, DNA-binding domain (C domain)

responsible for DNA-binding specificity and ER dimerization, and a C-terminal ligand-dependent transactivation (AF2) binding region.[2] Ligand binding to ER results in a conformational change regulating the receptor activity, DNA-binding and interactions with other proteins. The ligand-activated ER functions as a transcription factor, translocates to the nucleus, binds to responsive element (ERE) within target gene promoters, and stimulates gene transcription (extranuclear/nuclear signaling).[3, 4] Estrogens play an important role in mammary gland development and in the initiation and progression of breast cancer. ERα is the major ER subtype in the mammary epithelium and its importance in mammary gland biology and development has been confirmed in ERα (*Esr1*) knockout mice, which display grossly impaired ductal epithelial cell proliferation and branching.[5, 6]

Emerging evidence suggests that ER signaling is complex, involves cofactors, genomic actions, as well as extranuclear (cytoplasmic and membrane-mediated) actions [7-10]. Because of the nature and depth of the information available on estrogen mediated extranuclear actions in different cell types, only representative studies that involve ERα actions in breast cancer cells are included in this review. Here, we focus on summarizing the emerging key evidence for ERα extranuclear signaling in breast cancer progression and discuss the possibility of the targeting ERα extranuclear actions as an additional possible therapeutic target for preventing local and distant progression of estrogen-dependent breast cancer.

Molecular mechanisms of ER extra nuclear signaling

Kinase cascades

Emerging evidence suggests that ERα participates in extranuclear signaling via formation of a multiprotein complex collectively called a "*signalsome*".[11] Even though the complete repertoire of proteins present in the signalosome are not known, evidence suggests that ERα extranuclear signaling utilizes multiple cytosolic kinases. ERα-extranuclear signaling has been linked to rapid responses to E2 through stimulation of the Src kinase, mitogen-activated protein kinase (MAPK), protein kinase B (AKT), phosphatidylinositol- 3-kinase (PI3K), PKA and PKC pathways in the cytosol.[12, 13] The proto-oncogene c-Src is a multifunctional intracellular tyrosine kinase implicated in the regulation of a variety of processes including proliferation, differentiation, survival, and motility.[14] Src interacts with ERα and is overexpressed in breast tumors.[15] ERα exatranuclear actions also involve PKA signaling pathways and functional PKA signaling is needed for optimal activation of MAPK by E2.[16] Further, E2 -induced MAPK activation is shown to be mediated by PKC-delta/Ras pathway, that could be crucial for E2-dependent growth-promoting effects in the early stages of tumor progression.[17] Integrin linked kinase (ILK1) is another ERα interacting kinase; estrogen treatment enhances ILK activity and regulation of ER-ILK1 interaction is dependent on the PI3K pathway.[18]

Growth factor signaling

Growth factor receptors EGFR, ErbB2 and IGFR tether ERα to the plasma membrane and are involved in E2 biological actions by interacting with ER signalosome.[19] Growth factors promote the formation of a multi-protein complexes leading to the initiation of MAPK and PI3K signaling pathways in breast cancer cells.[20] Activation of the PI3K-AKT pathway has been shown to be an essential step in the estrogenic action of growth factors.[21] Signal transducer and activator of transcription (STAT) family of transcription factors play an important role in oncogenesis and signaling crosstalk occurs between ERα, c-Src, EGFR, and STAT5 in ERα positive breast cancer cells and STAT5 plays an integral role in E2-stimulated proliferation.[22] ERα also interacts with STAT3 and cross-talk between ERα and STAT3 play an important role in leptin-induced STAT3 activation.[23] The ILK1 axis is the major signaling node linking integrins and growth factor signaling to a variety of

cellular responses regulated by estrogens. ERα interacts with ILK1 enzyme [24] and ILK1 was identified as a novel interacting protein of ERα-coregulator PELP1 [25] and ILK functions as a downstream effector of ERα extranuclear signaling, leading to cytoskeleton reorganization.

ERα modifications

ERα undergoes several post-translational modifications including methylation, acetylation, phosphorylation, palmitoylation and S-nitrosylation affecting receptor subcellular localization, stability and ER extranuclear actions. Protein arginine N-methyltransferase 1 (PRMT1) transiently methylates arginine 260 located in the DNA-binding domain of ERα facilitating the interaction of ERα with p85 subunit of PI3K and Src, resulting in ERα extranuclear actions both in normal and malignant epithelial breast cells.[26] S-Palmitoylation, a reversible addition of palmitate on non-N-terminal Cys residues is catalyzed by palmitoyl acyl transferase (PAT), facilitates ERα localization to the plasma membrane. Thus enhancing the ERα interaction with adaptor proteins and kinases and activation of the AKT and MAPK pathways.[27] ERα and its coregulator's phosphorylation occurs on tyrosine and serine/threonine residues and such phosphorylation facilitating ERα extranuclear action leading to activation of the AKT pathway.[28] mTor and MAPK contribute to ERα activation via Serine 167 phosphorylation which has been associated with the development of therapeutic resistance.[29] Serine305 phosphorylation of ER by protein kinase A associates with tamoxifen sensitivity.[30] Nitrioxide (NO) can modify ERα via S-nitrosylation at cysteine residue resulting in selective inhibition of DNA-binding of ERα to ERE within target gene promoters. Suggesting, the interaction between NO and ERα favors activation of extranuclear actions and signaling pathways of ERα.[31] ERα forms a complex with histonedeacetylase (HDAC) 6 and tubulin at the plasma membrane in ligand dependent manner and promotes rapid deacetylation of tubulin of breast cancer cells. Estrogen-dependent tubulin deacetylation is another mechanism of ER extranuclear actions, and may potentially contributes to the aggressiveness of ERα-positive breast cancer cells.[32]

Adaptor molecules

Estrogen is shown to utilize several adaptor molecules to couple ERα with the growth factor signaling axis. Hormonal signaling promotes association of ERα with adaptor protein Shc, which couples additional needed signaling molecules such as Src and growth factor receptors.[19] Cytoskeltal associate protein p130Cas, another adaptor protein that associates with ERα signalosome, in a hormonal dependent manner. Overexpression of p130Cas increases estrogen mediated c-Src and MAPK activities.[33] Recent studies identified ERα coregulator PELP1, a scaffolding protein coupling ERα with Src kinase leading to activation of the cytosolic kinase pathways including MAPK and AKT. While all the components of the ERα signalosome have yet to be identified, emerging studies suggest that ERα, PELP1 and Src kinase represent key components that facilitating ERα extranuclear signaling.[34] Using transgenic mouse model that uniquely express PELP1 in the cytoplasm (MMTV_PELP-1cyto mice), it was demonstrated that cytoplasmic localization of ERα coregulator has potential to enhance ERα extranuclear signaling.[35] Metastatic tumor antigen 1 (MTA1), an ER coregulator protein and the naturally occurring short form of MTA1 (MTA1s) is reported to localize in the cytoplasm, sequesters ERα in the cytoplasm, and thus enhance ER extranuclear responses.[36]

Biological functions of ER extranuclear actions

ERα extranuclear actions in gene transcription

Several elegant studies investigated the impact of estrogen mediated extranuclear initiated pathways on global gene expres-

sion by using estrogen-dendrimer conjugates (EDCs).[37-40] EDCs are nanoparticles, coated with estradiol (E_2) through a 17α-phenylethynyl unit, have a binding affinity similar to estrogen, uniquely localize in the membrane/cytoplasm, and preferably activate ERα extranuclear signaling.[41, 42] Genome-wide cDNA microarray analysis revealed approximately 25% E2 target genes as EDC responsive. These studies using various assays and pharmacological inhibitors demonstrated that extranuclear signaling cascades have the potential to elicit gene stimulation.[39] Aromatase plays a critical role in breast cancer development by converting androgen to estrogen. Estrogen induces aromatase expression without direct binding of ERα to the aromatase promoter and E2 induction could be suppressed by the MAPK inhibitor or growth factor signaling inhibitor. The results from this study suggested that E2 up-regulates aromatase expression by ERα extranuclear actions via crosstalk with growth factor-mediated pathways.[43] Estrogen mediated extranuclear actions also promote phosphorylation of several key ERα transcriptional coregulators such as SRC3 and PELP1, thus enhancing their recruitment to target gene promoters and such actions implicate that ER extranuclear signaling may have downstream genomic roles via coactivator signaling.[44, 45] Estrogen induced transactivation of a STAT-regulated promoter requires MAPK, Src, and PI3K activity. These results implicate ER mediated extranuclear actions in nuclear transcriptional activation of STAT target genes.[46] E2 induces rapid nuclear translocation of MAPK together with cAMP response element binding protein leading to transcriptional activation of gene responsive to cAMP response element binding protein.[47] Estrogen mediated extracnuclear actions crosstalk with prolactin signaling results in enhanced activity of activating protein 1 and induction of c-fos gene in breast cancer cells.[48] Collectively, this evolving evidence implicates that inputs from ERα extranuclear pathways in regulating the gene expression of breast cancer cells.

ER extranuclear actions in cytoskeletal remodeling and metastasis

Clinically, estrogen has long been recognized to enhance the development and progression of ERα positive breast cancers. Several studies report a positive effect of ERα signaling on motility [49, 50] as many metastatic tumors retain ERα;[51] >80% of lymph node metastases and 65-70% of distant metastases maintain ERα expression.[52, 53] A correlation between ERα-positive tumors and development of bone metastasis has been observed clinically.[54, 55] Similarly, ERα-mediated signaling enhances lung metastasis by promoting host-compartment response.[56] Metastases spawned by malignant tumors that have acquired increased invasiveness are responsible for almost all breast cancer-related morbidity and mortality. Cancer cell metastasis is a multistage process involving invasion into surrounding tissue, intravasation, transit in the blood or lymph, extravasation, and growth at a new site; many of these steps require cell motility. This invasive phenotype, characterized by both the loss of cell-cell interactions and increased cellular motility, is driven by cycles of actin polymerization, cell adhesion and acto-myosin contraction.

Tumor cell motility is an essential step in metastasis allowing cancer cells to spread through tissues and migrate to distant organs. Endocrine therapy has also been shown to have a positive effect on the treatment of advanced metastatic disease.[57] Recent mechanistic studies have increased our understanding and highlight a role of estrogen-induced rapid ER extra-nuclear signaling in facilitating the metastatic process in breast cancer patients and may provide new targets for therapeutic interventions. ERα activation, by estrogen, induces key features of motile cells including rapid cytoskeletal reorganization and the development of specialized structures. Estrogen triggers rapid and dynamic actin cytoskeleton remodeling leading to increased breast cancer cell horizontal migration and invasion of three-dimensional matrices via the $G\alpha_{13}$/RhoA/ROCK/moesin cascade.[58] Estro-

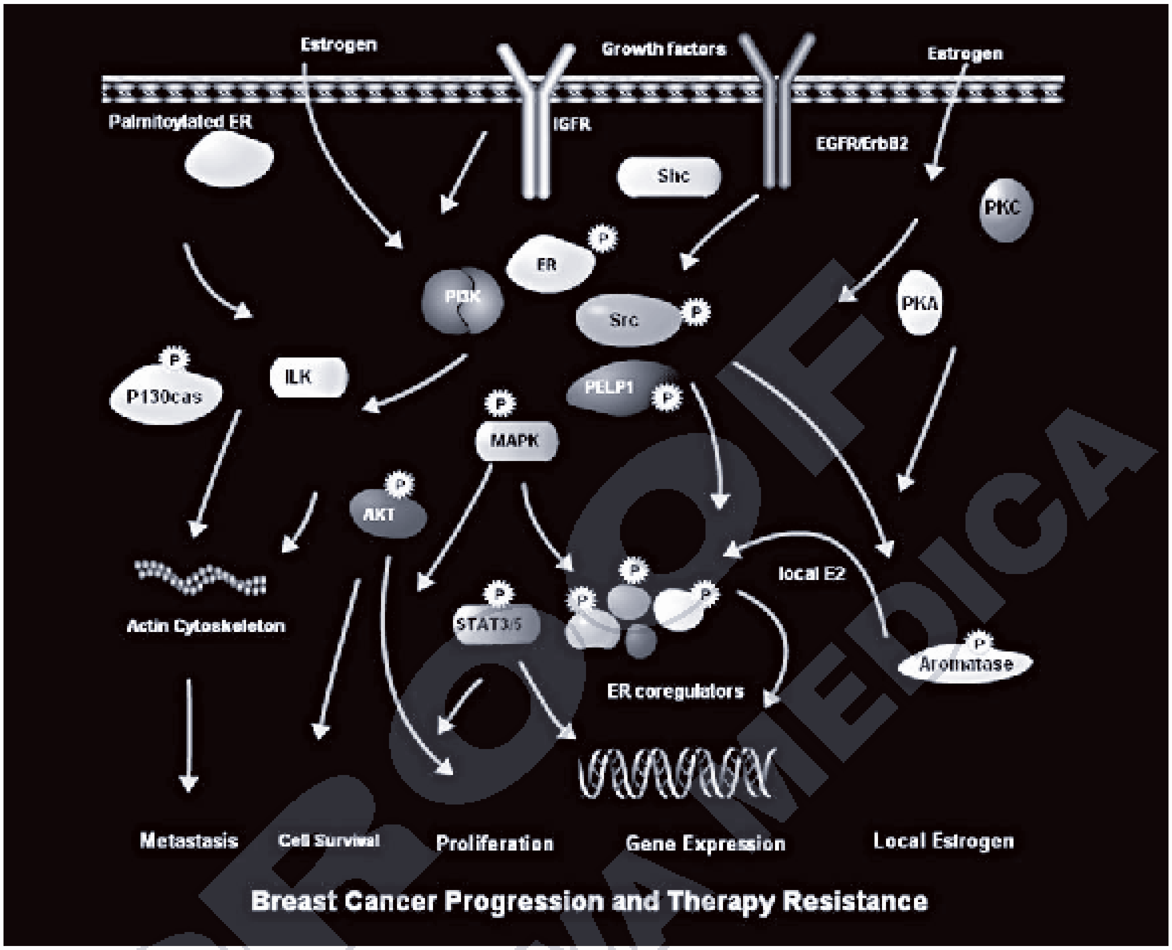

Figure 1.—Schematic representation of the current understanding of ER extranuclear signaling. Estrogen and growth factors promote ER complex formation with growth factors signaling components and cytosolic kinases that lead to activation of a number of pathways including MAPK, PI3K and AKT. Extranuclear pathways influence several biological functions including cell survival, proliferation and motility. Deregulation of ER extranuclear signaling will have implications in tumor cell metastasis and tumor progression.

gen-induced effects depend on the rapid recruitment and activation of the actin-binding protein, moesin, and the interaction of ERα with the G protein $G\alpha_{13}$, which results in the recruitment of the small GTPase RhoA, subsequent activation of its downstream effector Rho-associated kinase-2 (ROCK-2) and moesin phosphorylation.[58]

Recent studies also showed that estrogen-mediated extranuclear signaling promotes formation of signaling complexes containing PELP1, ERα, Src, and ILK1; signaling from this axis plays important roles in promoting cytoskeletal rearrangements, motility and metastasis.[25] Extranuclear actions of estrogen facilitate the activation of ILK via the PI3K pathway and inhibition of ILK functions significantly affected the estrogen-mediated migratory potential of breast cancer cells. The proposed signaling pathway, ERα ◊ PELP1 ◊ PI3K ◊ ILK ◊ CDC42, contributes to estrogen-mediated cytoskeleton rearrangements.[25] Emerging data regarding the impact of extranuclear signaling of estrogen on cytoskeletal organization suggests, ER-mediated control over cellular movement and invasion related to the catastrophic metastatic events in patients. Collectively, these results may in part explain carcinogenic actions and enhanced meta-

static behavior of estrogen-dependent, ER-positive breast cancer seen clinically.

ERα extranuclear actions in cell survival and proliferation

The use of novel ligands with the ability to uniquely activate extranuclear signals demonstrated the distinct biological outcomes of the extranuclear pathway.[42] Estrogen-dendrimer conjugate (EDC) which are excluded from the nucleus, verified ERα mediated extranuclear actions stimulates endothelial cell proliferation and migration via ERα direct interaction with Gαi and endothelial NOS (eNOS) activation.[59] Estrogen promotes ternary complex formation of ER with Src- and PI3K and the resulting pathways converge on cell cycle progression leading to estrogen induced S-phase entry.[36] Estrogen triggers cellular proliferation and survival through the activation of MAPK and AKT pathways respectively. Estrogen stimulation of cyclin D1 gene through ERK or PI3K activation promotes G1/S cell cycle progression in breast cancer cells.[60] Estrogen-induced growth of breast and lung cancer cells *in vitro* correlated closely with acute hormonal activation of MAPK signaling.[61] Ligand stimulation causes ERα to dissociate from caveolin-1 allowing the activation of signals to promote cellular proliferation.[62-64] A recent study demonstrated that ERα promotes transcription of Bcl-2 via PI3K-AKT cross-talk leading to enhanced cell survival.[65]

Significance of ER extranuclear signaling axis in breast cancer progression

Although much is known about ERα genomic actions, the pathobiology of ER extranuclear actions remains unknown. Some evidence suggests that the extranuclear effects of estrogen can regulate different cellular processes, such as proliferation, survival, apoptosis and differentiation functions in diverse cell-types, including breast cancer cells.[66] *In situ* estrogen production by aromatase conversion from androgens plays an important role in breast tumor progression. ERα mediated extranuclear signaling enhances aromatase enzymatic activity via activation of the Src enzyme. These results suggested a possible autocrine loop between E_2 and aromatase activity in breast cancer cells and implicate ERα actions in tumor progression.[67] Molecular adaptors such as PELP1 which couple ERα to cytosolic signaling axis may play a role in breast tumorigenesis via activation of ERα extranuclear signaling pathways.[68] Since breast tumors overexpress Src kinase, deregulation of PELP1 seen in breast tumors can contribute to activation of Src, leading to the progression to metastasis. ERα coregulator PELP1 acts as a scaffolding protein coupling the ERα with Src kinase leading to activation of the ER-Src-MAPK pathway.[69] Extranuclear expression of ER/PR occurs frequently in ERα-positive/PR-negative and ER-negative/PR-positive tumors, and in these cases evidence implicates nuclear receptor crosstalk with the PI3K/AKT signaling pathway whose activation by ErbB2 overexpression contributes to the growth of some breast cancers.[70] Dysregulation of ErbB2in breast cancer cells enhances the expression of MTA1s, promotes the cytoplasmic sequestration of ERα and stimulates malignant phenotypes. These study findings implicate that the regulation of the cellular localization of ERα by MTA1s represents a mechanism for enhancing ERα extranuclear actions by nuclear exclusion.[36] Methylated ERα is only present in the cytoplasm and arginine methylation is reversed by the demethylase JMJD6, suggesting deregulation of arginine methtylation and demethylation will have consequences in activation of ERα extranuclear actions. In addition, arginine methylation also regulates the balance between coactivator complex assembly and disassembly. Since methylation enzymes such PRMT1 and CARM1 are dysregulated in estrogen-dependent cancers, they are implicated in promoting ER extranuclear signaling [71].

ERα extranuclear actions and hormonal therapy resistance

ERα crosstalk with growth factor signaling play an important role in enhancing ER ex-

tranuclear signaling. ErbB2 is an oncogene that has been shown to be over expressed, amplified, or both, in breast tumors. ER expression occurs in ~50% ErbB2 positive breast cancers and crosstalk between the ERα and ErbB2 pathways promotes endocrine therapy resistance.[72, 73] ERα-coregulator PELP1 plays an essential role in ERα extranuclear actions by coupling ERα with Src and PI3K pathways.[69, 74] PELP1 interacts with growth factor signaling components and participates in ligandindependent activation of ERα.[75, 76] In our previous studies, we found that in a subset of breast tumors PELP1 is predominantly localized in the cytoplasm, breast cancer model cells mimicking PELP1 cytoplasmic expression showed resistance to tamoxifen via excessive activation of c-Src signaling axis.[45] ERα extranuclear pathways have been shown to modify ERα or its coactivators by phosphorylation, resulting in the altered topology of ERα and its coregulator proteins and eventually leading to ligand-independent activation or differential responses to selective estrogen receptor modulators.[9, 12] Forced expression of constitutively active AKT in MCF-7 cells promotes estrogen-independent growth as well as tamoxifen response.[77] Overexpression of the ERα coactivator SRC3 promoted high tumor incidence, which is associated with the activation of the PI3K-AKT pathway.[78] Extranuclear expression of ERα-coregulators such as PELP1 correlates with increases in extranuclear signaling and has the potential to be used as a determinant of hormone sensitivity or vulnerability.[35] Recent findings suggest that ILK1 interacts with PELP1 [25] and that such interactions enhance ILK1-kinase activity. Since PELP1 expression is commonly deregulated in many hormone-responsive tissues,[79] the PELP1-ILK1 interaction is likely to have significant implications in tumor cell survival and therapy resistance. In cells developing resistance to estrogen deprivation by anti-estrogens/aromatase inhibitors, an increased association of ERα with c-Src and EGFR occurs. Further, these conditions promote translocation of ERα out of the nucleus and into the cytoplasm and cell membrane. This study suggested that secondary resistance to hormonal therapy results in usage of both IGFR and EGFR for ERα extranuclear signaling.[80]

Therapeutic potential of targeting ER extranuclear actions

ERα extranuclear pathways promote hormone-mediated proliferation and survival of breast tumors making them a promising target for anti-tumor therapy via the combination of anti-estrogens and ER extranuclear signaling blockers.[61] ERα extranuclear actions involve kinase cascades and post-translational modifications which can be reversed by pharmacological inhibitors currently in clinical trials. Inhibitors of EGFR, ERBB2, MAPK and AKT pathways could be used to block ER extranuclear signaling in ERα positive tumors that exhibit deregulation of these pathways.[72, 81] Pharmacological inhibition of Src using dasatinib inhibits estrogen-mediated extranuclear actions and reduces estrogen-mediated migratory potential suggestive of the therapeutic value of dasatinib in blocking ER-positive metastases.[25] ER extranuclear signaling utilizes the ILK axis and ILK inhibitor (QLT-0267) in combination with docetaxel exhibited synergistic effects on reducing the viability of breast cancer cells.[82] ILK inhibitors also have the potential to down regulate the ILK-mediated EMT phenotype and tumorigenesis. ER extranuclear actions mediate activation of STAT3/5, and ERα-STAT crosstalk is implicated in breast tumorigenesis and therapy resistance.[46] STAT inhibitors currently in clinical trials could be used to block ER extranuclear actions. Since arginine methylation is involved in ERα extranuclear signaling, this modification is a possible therapeutic target by using guanidine nitrogen-substituted peptides or the thioglycolic amide, RM65.[83, 84] As both ERα genomic and extranuclear signaling are involved in breast tumorigenesis and therapy resistance, a therapeutic approach to inhibit ER extranuclear actions along with current endocrine therapies could have better therapeutic efficacy and delay the on-set of hormonal resistance in advanced breast tumors.

Conclusions

Emerging evidence suggests, in addition to genomic functions, ER participates in extranuclear rapid signaling via the formation of signaling complexes in the cytoplasm with both physiological and pathological consequences. The ability of ERα to participate in extranuclear actions, cytoplasmic localization of ERα and ERα co-activators in breast tumors and ERα -growth factor signaling crosstalk, strongly suggests that ERα extranuclear actions play a key role in breast tumor pathogenesis and development of therapy resistance. Future studies identifying the molecular mechanisms of ERα extranuclear signaling and components of the signalosome contributing to ERα extranuclear signaling as well as to examining the prognostic / diagnostic significance of ERα extranuclear signaling using a larger tumor sample size are warranted. Further, elucidation of the normal and pathological roles of ERα extranuclear signaling will have important implications for breast cancer treatment and in the development of next generation estrogen receptor modulators.

Riassunto

Vie del segnale extranucleari degli estrogeni: ruolo nella progressione e nella meta statizzazione del cancro della mammella

Il recettore per gli estrogeni (ERα) è implicato nella progressione del cancro mammario. Le ormonoterapie che bloccano le funzioni dell'ER o la produzione locale e sistemica di estrogeni sono attualmente utilizzate nel trattamento del cancro della mammella positivo per i recettori degli estrogeni. L'ormonoterapia mostra effetti positivi, tuttavia spesso si instaura una resistenza iniziale o acquisita alle terapie endocrine, e le neoplasie recidivano con metastasi a distanza. Secondo recenti evidenze, in aggiunta alle ben studiate funzioni nucleari l'ERα partecipa anche alle vie del segnale extranucleari che coinvolgono le componenti delle vie del segnale del fattore di crescita, le molecole adattatrici e la stimolazioni di kinasi citosoliche. Le vie extranucleari dell'ERα possono attivare la trascrizione genica, modulare il citoscheletro, e promuovere la proliferazione, la sopravvivenza delle cellule tumorali e favorire lo sviluppo di metastasi. L'ERα citoplasmatico/di membrane viene riscontrato in un sottogruppo di tumori della mammella e l'espressione delle componenti extranucleari dell' ERα è deregolata nei tumori. Le azioni extranucleari dell'ER si stanno dimostrando un bersaglio importante per il controllo tumorigenico e metastatico. L'inibizione delle azioni extranucleari dell' ERα ha la possibilità di prevenire la progressione del tumore della mammella e potrebbe essere utile nella prevenzione delle metastasi ERα positive. In questa revisione, autori riassumono i risultati dei recenti studi sul ruolo delle azioni extranucleari ERα mediate nella tumorigenesi e metastatizzazione del tumore della mammella.

Parole chiave: Estrogeni - Tumore del seno - Tumori, metastasi.

References

1. Warner M, Nilsson S, Gustafsson JA. The estrogen receptor family. Curr Opin Obstet Gynecol 1999;11:249-54.
2. Kumar V, Green S, Stack G, Berry M, Jin JR, Chambon P. Functional domains of the human estrogen receptor. Cell 1987;51:941-51.
3. McKenna NJ, Lanz RB, O'Malley BW. Nuclear receptor coregulators: cellular and molecular biology. Endocr Rev 1999;20:321-44.
4. McDonnell DP, Norris JD. Connections and regulation of the human estrogen receptor. Science 2002;296:1642-4.
5. Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci U S A 1993;90:11162-6.
6. Bocchinfuso WP, Korach KS. Mammary gland development and tumorigenesis in estrogen receptor knockout mice. J Mammary Gland Biol Neoplasia 1997;2:323-34.
7. McDonnell DP, Norris JD. Connections and regulation of the human estrogen receptor. Science 2002;296:1642-4.
8. Bjornstrom L, Sjoberg M. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. Mol Endocrinol 2005;19:833-42.
9. Carroll JS, Brown M. Estrogen receptor target gene: an evolving concept. Mol Endocrinol 2006;20:1707-14.
10. Yang LC, Zhang QG, Zhou CF, Yang F, Zhang YD, Wang RM *et al.* Extranuclear estrogen receptors mediate the neuroprotective effects of estrogen in the rat hippocampus. PLoS One 2010;5:e9851.
11. Levin ER. Integration of the extranuclear and nuclear actions of estrogen. Mol Endocrinol 2005;19:1951-9.
12. Hammes SR, Levin ER. Extranuclear steroid receptors: nature and actions. Endocr Rev 2007;28:726-41.
13. Fox EM, Andrade J, Shupnik MA. Novel actions of estrogen to promote proliferation: integration of cytoplasmic and nuclear pathways. Steroids 2009;74:622-7.
14. Trevino JG, Summy JM, Gallick GE. SRC inhibitors as potential therapeutic agents for human cancers. Mini Rev Med Chem 2006;6:681-7.
15. Russello SV, Shore SK. SRC in human carcinogenesis. Front Biosci 2004;9:139-44.

16. Belcher SM, Le HH, Spurling L, Wong JK. Rapid estrogenic regulation of extracellular signal- regulated kinase 1/2 signaling in cerebellar granule cells involves a G protein- and protein kinase A-dependent mechanism and intracellular activation of protein phosphatase 2A. Endocrinology 2005;146:5397-406.
17. Keshamouni VG, Mattingly RR, Reddy KB. Mechanism of 17-beta-estradiol-induced Erk1/2 activation in breast cancer cells. A role for HER2 AND PKC-delta. J Biol Chem 2002;277:22558-65.
18. Acconcia F, Manavathi B, Mascarenhas J, Talukder AH, Mills G, Kumar R. An inherent role of integrin-linked kinase-estrogen receptor alpha interaction in cell migration. Cancer Res 2006;66:11030-8.
19. Song RX, Barnes CJ, Zhang Z, Bao Y, Kumar R, Santen RJ. The role of Shc and insulin-like growth factor 1 receptor in mediating the translocation of estrogen receptor alpha to the plasma membrane. Proc Natl Acad Sci U S A 2004;101:2076-81.
20. Song RX, Fan P, Yue W, Chen Y, Santen RJ. Role of receptor complexes in the extranuclear actions of estrogen receptor alpha in breast cancer. Endocr Relat Cancer 2006;13 Suppl 1:S3-13.:S3-13.
21. Martin MB, Franke TF, Stoica GE, Chambon P, Katzenellenbogen BS, Stoica BA, McLemore MS, Olivo SE, Stoica A. A role for Akt in mediating the estrogenic functions of epidermal growth factor and insulin-like growth factor I. Endocrinology 2000;141:4503-11.
22. Fox EM, Bernaciak TM, Wen J, Weaver AM, Shupnik MA, Silva CM. Signal transducer and activator of transcription 5b, c-Src, and epidermal growth factor receptor signaling play integral roles in estrogen-stimulated proliferation of estrogen receptor-positive breast cancer cells. Mol Endocrinol 2008;22:1781-96.
23. Binai NA, Damert A, Carra G, Steckelbroeck S, Lower J, Lower R, Wessler S. Expression of estrogen receptor alpha increases leptin-induced STAT3 activity in breast cancer cells. Int J Cancer 2010;127:55-66.
24. Acconcia F, Kumar R. Signaling regulation of genomic and nongenomic functions of estrogen receptors. Cancer Lett 2006;238:1-14.
25. Chakravarty D, Nair SS, Santhamma B, Nair BC, Wang L, Bandyopadhyay A *et al.* Extranuclear functions of ER impact invasive migration and metastasis by breast cancer cells. Cancer Res 2010;70:4092-101.
26. Le RM, Treilleux I, Leconte N, Robin-Lespinasse Y, Sentis S, Bouchekioua-Bouzaghou K *et al.* Regulation of estrogen rapid signaling through arginine methylation by PRMT1. Mol Cell 2008;31:212-21.
27. Marino M, Ascenzi P, Acconcia F. S-palmitoylation modulates estrogen receptor alpha localization and functions. Steroids 2006;71:298-303.
28. Cheskis BJ, Greger J, Cooch N, McNally C, McLarney S, Lam HS *et al.* MNAR plays an important role in ERa activation of Src/MAPK and PI3K/Akt signaling pathways. Steroids 2008;73:901-5.
29. Yamnik RL, Holz MK. mTOR/S6K1 and MAPK/RSK signaling pathways coordinately regulate estrogen receptor alpha serine 167 phosphorylation. FEBS Lett 2010;584:124-8.
30. Kok M, Zwart W, Holm C, Fles R, Hauptmann M, Van't Veer LJ *et al.* PKA-induced phosphorylation of ERalpha at serine 305 and high PAK1 levels is associated with sensitivity to tamoxifen in ER-positive breast cancer. Breast Cancer Res Treat 2010. [Epub ahead of print]
31. Garban HJ, Marquez-Garban DC, Pietras RJ, Ignarro LJ. Rapid nitric oxide-mediated S-nitrosylation of estrogen receptor: regulation of estrogen-dependent gene transcription. Proc Natl Acad Sci U S A 2005;102:2632-6.
32. Azuma K, Urano T, Horie-Inoue K, Hayashi S, Sakai R, Ouchi Y, Inoue S. Association of estrogen receptor alpha and histone deacetylase 6 causes rapid deacetylation of tubulin in breast cancer cells. Cancer Res 2009;69:2935-40.
33. Cabodi S, Moro L, Baj G, Smeriglio M, Di SP, Gippone S, Surico N, Silengo L, Turco E, Tarone G, Defilippi P. p130Cas interacts with estrogen receptor alpha and modulates non-genomic estrogen signaling in breast cancer cells. J Cell Sci 2004;117:1603-11.
34. Song RX, Santen RJ. Membrane initiated estrogen signaling in breast cancer. Biol Reprod 2006;75:9-16.
35. Kumar R, Zhang H, Holm C, Vadlamudi RK, Landberg G, Rayala SK. Extranuclear coactivator signaling confers insensitivity to tamoxifen. Clin Cancer Res 2009;15:4123-30.
36. Kumar R, Wang RA, Mazumdar A, Talukder AH, Mandal M, Yang Z *et al.* A naturally occurring MTA1 variant sequesters oestrogen receptor-alpha in the cytoplasm. Nature 2002;418:654-7.
37. Kim SH, Katzenellenbogen JA. Hormone-PAMAM dendrimer conjugates: polymer dynamics and tether structure affect ligand access to receptors. Angew Chem Int Ed Engl 2006;45:7243-8.
38. Chambliss KL, Wu Q, Oltmann S, Konaniah ES, Umetani M, Korach KS *et al.* Non-nuclear estrogen receptor alpha signaling promotes cardiovascular protection but not uterine or breast cancer growth in mice. J Clin Invest 2010;120:2319-30.
39. Madak-Erdogan Z, Kieser KJ, Kim SH, Komm B, Katzenellenbogen JA, Katzenellenbogen BS. Nuclear and extranuclear pathway inputs in the regulation of global gene expression by estrogen receptors. Mol Endocrinol 2008;22:2116-27.
40. Zhang QG, Raz L, Wang R, Han D, De SL, Yang F *et al.* Estrogen attenuates ischemic oxidative damage via an estrogen receptor alpha-mediated inhibition of NADPH oxidase activation. J Neurosci 2009;29:13823-36.
41. Trogden BG, Kim SH, Lee S, Katzenellenbogen JA. Tethered indoles as functionalizable ligands for the estrogen receptor. Bioorg Med Chem Lett 2009;19:485-8.
42. Harrington WR, Kim SH, Funk CC, Madak-Erdogan Z, Schiff R, Katzenellenbogen JA *et al.* Estrogen dendrimer conjugates that preferentially activate extranuclear, nongenomic versus genomic pathways of estrogen action. Mol Endocrinol 2006;20:491-502.
43. Kinoshita Y, Chen S. Induction of aromatase (CYP19) expression in breast cancer cells through a nongenomic action of estrogen receptor alpha. Cancer Res 2003;63:3546-55.
44. Zheng FF, Wu RC, Smith CL, O'Malley BW. Rapid estrogen-induced phosphorylation of the SRC-3 coactivator occurs in an extranuclear complex containing estrogen receptor. Mol Cell Biol 2005;25:8273-84.
45. Vadlamudi RK, Manavathi B, Balasenthil S, Nair SS, Yang Z, Sahin AA, Kumar R. Functional implications of altered subcellular localization of PELP1 in breast cancer cells. Cancer Res 2005;65:7724-32.
46. Bjornstrom L, Sjoberg M. Signal transducers and activators of transcription as downstream targets of nongenomic estrogen receptor actions. Mol Endocrinol 2002;16:2202-14.
47. Dos Santos EG, Dieudonne MN, Pecquery R, Le M, V, Giudicelli Y, Lacasa D. Rapid nongenomic E2 effects on p42/p44 MAPK, activator protein-1, and cAMP response element binding protein in rat white adipocytes. Endocrinology 2002;143:930-40.

48. Gutzman JH, Nikolai SE, Rugowski DE, Watters JJ, Schuler LA. Prolactin and estrogen enhance the activity of activating protein 1 in breast cancer cells: role of extracellularly regulated kinase 1/2-mediated signals to c-fos. Mol Endocrinol 2005;19:1765-78.
49. Sisci D, Aquila S, Middea E, Gentile M, Maggiolini M, Mastroianni F *et al.* Fibronectin and type IV collagen activate ERalpha AF-1 by c-Src pathway: effect on breast cancer cell motility. Oncogene 2004;23:8920-30.
50. Thompson EW, Reich R, Shima TB, Albini A, Graf J, Martin GR *et al.* Differential regulation of growth and invasiveness of MCF-7 breast cancer cells by antiestrogens. Cancer Res 1988;48:6764-8.
51. Koenders PG, Beex LV, Langens R, Kloppenborg PW, Smals AG, Benraad TJ. Steroid hormone receptor activity of primary human breast cancer and pattern of first metastasis. The Breast Cancer Study Group. Breast Cancer Res Treat 1991;18:27-32.
52. Harrell JC, Dye WW, Allred DC, Jedlicka P, Spoelstra NS, Sartorius CA *et al.* Estrogen receptor positive breast cancer metastasis: altered hormonal sensitivity and tumor aggressiveness in lymphatic vessels and lymph nodes. Cancer Res 2006;66:9308-15.
53. Zheng WQ, Lu J, Zheng JM, Hu FX, Ni CR. Variation of ER status between primary and metastatic breast cancer and relationship to p53 expression*. Steroids 2001;66:905-10.
54. Koenders PG, Beex LV, Langens R, Kloppenborg PW, Smals AG, Benraad TJ. Steroid hormone receptor activity of primary human breast cancer and pattern of first metastasis. The Breast Cancer Study Group. Breast Cancer Res Treat 1991;18:27-32.
55. Wang J, Jarrett J, Huang CC, Satcher RL, Jr., Levenson AS. Identification of estrogen-responsive genes involved in breast cancer metastases to the bone. Clin Exp Metastasis 2007;24:411-22.
56. Banka CL, Lund CV, Nguyen MT, Pakchoian AJ, Mueller BM, Eliceiri BP. Estrogen induces lung metastasis through a host compartment-specific response. Cancer Res 2006;66:3667-72.
57. Utsumi T, Kobayashi N, Hanada H. Recent perspectives of endocrine therapy for breast cancer. Breast Cancer 2007;14:194-199.
58. Giretti MS, Fu XD, De RG, Sarotto I, Baldacci C, Garibaldi S *et al.* Extra-nuclear signalling of estrogen receptor to breast cancer cytoskeletal remodelling, migration and invasion. PLoS One 2008;3:e2238.
59. Chambliss KL, Wu Q, Oltmann S, Konaniah ES, Umetani M, Korach KS *et al.* Non-nuclear estrogen receptor alpha signaling promotes cardiovascular protection but not uterine or breast cancer growth in mice. J Clin Invest 2010;120:2319-30.
60. Fu XD, Cui YH, Lin GP, Wang TH. Non-genomic effects of 17beta-estradiol in activation of the ERK1/ERK2 pathway induces cell proliferation through up-regulation of cyclin D1 expression in bovine artery endothelial cells. Gynecol Endocrinol 2007;23:131-7.
61. Pietras RJ, Marquez DC, Chen HW, Tsai E, Weinberg O, Fishbein M. Estrogen and growth factor receptor interactions in human breast and non-small cell lung cancer cells. Steroids 2005;70:372-81.
62. Acconcia F, Ascenzi P, Bocedi A, Spisni E, Tomasi V, Trentalance A *et al.* Palmitoylation-dependent estrogen receptor alpha membrane localization: regulation by 17beta-estradiol. Mol Biol Cell 2005;16:231-7.
63. Galluzzo P, Caiazza F, Moreno S, Marino M. Role of ERbeta palmitoylation in the inhibition of human colon cancer cell proliferation. Endocr Relat Cancer 2007;14:153-67.
64. Marino M, Ascenzi P. Membrane association of estrogen receptor alpha and beta influences 17beta-estradiol-mediated cancer cell proliferation. Steroids 2008;73:853-8.
65. Bratton MR, Duong BN, Elliott S, Weldon CB, Beckman BS, McLachlan JA *et al.* Regulation of ERalpha-mediated transcription of Bcl-2 by PI3K-AKT crosstalk: implications for breast cancer cell survival. Int J Oncol 2010;37:541-50.
66. Acconcia F, Kumar R. Signaling regulation of genomic and nongenomic functions of estrogen receptors. Cancer Lett 2006 [Epub ahead of print].
67. Catalano S, Barone I, Giordano C, Rizza P, Qi H, Gu G, Malivindi R, Bonofiglio D, Ando S. Rapid estradiol/ERalpha signaling enhances aromatase enzymatic activity in breast cancer cells. Mol Endocrinol 2009;23:1634-45.
68. Vadlamudi RK, Kumar R. Functional and biological properties of the nuclear receptor coregulator PELP1/MNAR. Nucl Recept Signal 2007;5:e004.
69. Barletta F, Wong CW, McNally C, Komm BS, Katzenellenbogen B, Cheskis BJ. Characterization of the interactions of estrogen receptor and MNAR in the activation of cSrc. Mol Endocrinol 2004;18:1096-108.
70. Kim R, Kaneko M, Arihiro K, Emi M, Tanabe K, Murakami S *et al.* Extranuclear expression of hormone receptors in primary breast cancer. Ann Oncol 2006;17:1213-20.
71. Teyssier C, Le RM, Sentis S, Jalaguier S, Corbo L, Cavailles V. Protein arginine methylation in estrogen signaling and estrogen-related cancers. Trends Endocrinol Metab 2010;21:181-9.
72. Schiff R, Massarweh SA, Shou J, Bharwani L, Arpino G, Rimawi M, Osborne CK. Advanced concepts in estrogen receptor biology and breast cancer endocrine resistance: implicated role of growth factor signaling and estrogen receptor coregulators. Cancer Chemother Pharmacol 2005;56 Suppl 1:10-20.
73. Marcom PK, Isaacs C, Harris L, Wong ZW, Kommarreddy A, Novielli N *et al.* The combination of letrozole and trastuzumab as first or second-line biological therapy produces durable responses in a subset of HER2 positive and ER positive advanced breast cancers. Breast Cancer Res Treat 2007;102:43-9.
74. Wong CW, McNally C, Nickbarg E, Komm BS, Cheskis BJ. Estrogen receptor-interacting protein that modulates its nongenomic activity-crosstalk with Src/Erk phosphorylation cascade. Proc Natl Acad Sci U S A 2002;99:14783-8.
75. Manavathi B, Nair SS, Wang RA, Kumar R, Vadlamudi RK. Proline-, glutamic acid-, and leucine-rich protein-1 is essential in growth factor regulation of signal transducers and activators of transcription 3 activation. Cancer Res 2005;65:5571-7.
76. Nagpal J, Nair S, Pothana S, tekmal R, Kumar R, Vadlamudi R. Growth factor regulation of PELP1/MNAR functions: Role of PKA-dependent phosphorylation; 2006:2933.
77. Faridi J, Wang L, Endemann G, Roth RA. Expression of constitutively active Akt-3 in MCF-7 breast cancer cells reverses the estrogen and tamoxifen responsivity of these cells in vivo. Clin Cancer Res 2003;9:2933-9.
78. Torres-Arzayus MI, Font de MJ, Yuan J, Vazquez F, Bronson R, Rue M *et al.* High tumor incidence and activation of the PI3K/AKT pathway in transgenic mice define AIB1 as an oncogene. Cancer Cell 2004;6:263-74.
79. Chakravarty D, Tekmal RR, Vadlamudi RK. PELP1: A novel therapeutic target for hormonal cancers. IUBMB Life 2010;62:162-9.
80. Song RX, Chen Y, Zhang Z, Bao Y, Yue W, Wang JP *et al.* Estrogen utilization of IGF-1-R and EGF-R to

signal in breast cancer cells. J Steroid Biochem Mol Biol 2010;118:219-30.
81. Gururaj AE, Rayala SK, Vadlamudi RK, Kumar R. Novel mechanisms of resistance to endocrine therapy: genomic and nongenomic considerations. Clin Cancer Res 2006;12:1001s-1007s.
82. Kalra J, Warburton C, Fang K, Edwards L, Daynard T, Waterhouse D *et al.* QLT0267, a small molecule inhibitor targeting integrin-linked kinase (ILK), and docetaxel can combine to produce synergistic interactions linked to enhanced cytotoxicity, reductions in P-AKT levels, altered F-actin architecture and improved treatment outcomes in an orthotopic breast cancer model. Breast Cancer Res 2009;11:R25.
83. Spannhoff A, Machmur R, Heinke R, Trojer P, Bauer I, Brosch G *et al.* A novel arginine methyltransferase inhibitor with cellular activity. Bioorg Med Chem Lett 2007;17:4150-3.
84. Lakowski TM, 't HP, Ahern CA, Martin NI, Frankel A. N(eta)-Substituted Arginyl Peptide Inhibitors of Protein Arginine N-Methyltransferases. ACS Chem Biol 2010 [Epub ahead of print].

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Role of Estrogen Receptor Signaling in Breast Cancer Metastasis

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Abstract

Metastatic breast cancer is a life-threatening stage of cancer and is the leading cause of death in advanced breast cancer patients. Estrogen signaling and the estrogen receptor (ER) are implicated in breast cancer progression and the majority of the human breast cancers start out as estrogen-dependent. Accumulating evidence suggests that ER signaling is complex, involves coregulatory proteins and extranuclear actions. ER-coregulatory proteins are tightly regulated under normal conditions with miss-expression primarily reported in cancer. Deregulation of ER-coregualtors or ER extra-nuclear signaling has potential to promote metastasis in ER-positive breast cancer cells. This review summarizes the emerging role of ER signaling in promoting metastasis of breast cancer cells, discusses the molecular mechanisms by which ER signaling contribute to metastasis, and explores possible therapeutic targets to block ER driven metastasis.

Introduction

The steroid hormone, estradiol plays an important role in the progression of breast cancer and a majority of the human breast cancers start out as estrogen-dependent and express the estrogen receptor (ER). The biological effects of estrogen are mediated by its binding to one of the structurally and functionally distinct ERs (ERα and ERβ) [1]. Endocrine therapy using Tamoxifen, a selective estrogen receptor modulator [2], and aromatase inhibitors, which ablate peripheral estrogen synthesis, has been shown to substantially improve disease-free survival [3]. Endocrine therapy has also been shown to have a positive effect on the treatment of ER-positive breast cancer [4]. Despite these positive effects, initial or acquired resistance to endocrine therapies frequently occurs with tumors recurring as metastatic. Tumor metastasis comprises a series of discrete biological processes that moves tumor cells from the primary neoplasm to a distant location [5] and involves a multi-step cascade of coordinated cell adhesion and contractility as well as proteolytic remodeling of the extra-cellular matrix (ECM) [6, 7]. Even though substantial information is available on the process of metastasis, the molecular basis of breast cancer progression to metastasis and the role of ERα signaling in this process remain poorly understood. A few early studies suggested a negative effect of ERα signaling on motility and invasion of cells [8, 9], while several recent studies showed a positive effect of ER signaling on motility [10-14]. In this review, we summarized the emerging evidence for the role of ERα signaling in breast cancer progression to metastasis and discuss the possibility of targeting ERα signaling crosstalk with cytosolic kinases as a possible additional therapeutic target for treating / preventing ER-positive metastatic breast cancer.

ERα signaling mechanisms

ERα is the major ER subtype in the mammary epithelium and plays a critical role in mammary gland biology as well as in breast cancer progression [15, 16]. The ERα comprises an N-terminal AF1 domain, a DNA-binding domain, and a C-terminal ligand-binding region that contains an AF2 domain [17]. Upon the binding of estrogen to ERα, the ligand-activated ERα translocates to the nucleus, binds to the responsive element in the target gene promoter, and stimulates gene transcription (genomic/nuclear signaling) [18, 19]. Emerging evidence suggests that ER signaling is complex, involving coregulatory proteins and also genomic actions and extranuclear actions [20, 21].

Multi-protein complexes containing coregulators assemble in response to hormone binding and activate ER-mediated transcription [18]. The ERα transcriptional outcome is regulated by dynamic chromatin modifications of the histone tails and the ligand-bound ERα facilitates these modifications via coregulator recruitment [22]. For example, coactivators like SRC-1, amplified in breast cancer (AIB1) and CBP have been shown to possess histone acetyltransferase activity, whereas corepressors, such as NCOR and MTA1, are associated with histone deacetylases [20, 23]. It is generally accepted that some of the diverse functions of E2 depend on differential recruitment of coregulators to the E2-ER complex [24]. Even though coregulators modulate ER functions, each coregulator protein appears to play an important but not overlapping function *in vivo* [25-27].

Emerging findings suggest that ER-coregulator proteins have potential to be differentially expressed in malignant tumors, and that their functions may be altered, leading to tumor progression [28]. *In vivo* studies using wild-type (WT) and SRC3/AIB1$^{-/-}$ mice harboring the mouse mammary tumor virus-polyomavirus middle T (PyMT) transgene (Tg) revealed that AIB1 knock down significantly reduces lung metastasis but not mammary tumorigensis. Compared

with *WT*/*PyMT* mice, Tg *SRC-1*$^{-/-}$/*PyMT* mice had intravasation of mammary tumor cells. In addition, the frequency and extent of lung metastasis were drastically lower in the Tg mice than in the WT mice [29]. Another study using Tg *SRC-1*$^{-/-}$ mice reported that deficiency of SRC-1 coregulator increases MMTV-neu-mediated tumor latency and differentiation-specific gene expression and decreases metastasis [30]. Collectively, these emerging findings implicate the role of the ERα-coregulator associated activities/functions in breast cancer metastasis.

ERα **genomic actions and metastasis**

Within the last decade, research has provided substantial data to suggest that alteration in cellular concentration or genetic dysfunction of coregulators can contribute to a pathologic outcome by modulating ER genomic actions and has potential to drive cancer cell proliferation and metastasis [31]. Loss of the epithelial adhesion molecule E-cadherin is implicated with a critical role in metastasis by disrupting intercellular contacts–an early step in metastatic dissemination [32]. Functional or transcriptional loss is commonly associated with an invasive and poorly differentiated phenotype [33]. Deregulation of ER-coregulator signaling can lead to aberrant expression of Snail, resulting in the loss of expression of E-cadherin and invasive growth. For example, MTA1, a commonly deregulated coregulator in breast cancer promotes transcriptional repression of ER, leading to metastatic progression [34]. The ERα coregulator (AIB1) amplified in breast cancer has been shown to promote breast cancer metastasis by activation of PEA3-mediated matrix metalloproteinase 2 (MMP2) and MMP9 expression [35]. SRC-1, another ER coregulator, has also been shown to promote breast cancer invasiveness and metastasis by coactivating PEA3-mediated Twist expression [36]. Recent studies have found deregulation of the ER coregulator PELP1 in invasive and metastatic breast tumors [37, 38]. Recent studies

using PELP1 overexpression and knock down demonstrated that PELP1 plays an important role in ERα-positive metastasis [10]. Collectively, these studies indicate that ERα and ER-coregulators modulate expression of genes involved in metastasis.

ERα **extra-nuclear actions and metastasis**

Emerging evidence suggests that the ERα participates in extranuclear signaling [39]. ERα activation, by E2, induces key features of motile cells including rapid cytoskeletal reorganization and the development of specialized structures including fillopodia and ruffles [37]. To establish the role of E2-mediated extranuclear actions, researchers developed E2-Dendrimers (EDC), which are nanoparticles coated with estrogen. These EDC uniquely localize in the membrane and cytoplasm, preferably activating ERα -extranuclear signaling. Using these EDC, researchers have demonstrated that ERα extranuclear pathways have distinct biological outcomes [40]. Our laboratory using EDC provided further evidence that ERα -extranuclear signaling has the potential to contribute to the breast cancer cell motility (Figure 1) [10]. ERα -extranuclear signaling promotes stimulation of the Src kinase, mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and protein kinase C pathways in the cytosol (10, 11). Recent studies identified PELP1 as one of the components of the ERα signalosome in the cytoplasm and estrogen mediated extranuclear signaling promotes cytoskeleton reorganization via ER-Src-PELP1-PI3K-ILK1 pathway [10]. Many of the kinases activated by ERα extra-nuclear signaling are implicated in breast cancer metastasis. For example, ERK and protein kinase B (AKT) phosphorylation play important roles in breast cancer cell migration [14], and Src and ILK1 kinases play critical roles in invasion and metastasis of breast cancer cells [41, 42].

In addition to ERα interactions with cytosolic kinases, few other mechanisms by which the ERα activates extranuclear signaling have been reported. Membrane-bound ERα has been reported to be associated with growth factor receptors such as IGF-1R, EGFR, and HER2; and such interactions plays a role in cytoskeleton reorganization [43]. Dysregulation of HER2 in breast cancer cells enhances the expression of an isoform of MTA1 (MTA1s), which promotes the cytoplasmic sequestration of ERα leading to constitutive activation of MAPK. These study findings implicate the regulation of the cellular localization of ERα by MTA1s as a mechanism for enhancing ERα extranuclear actions by nuclear exclusion [44]. Recent studies also found that the ERα was methylated via post-translational modifications and methylated ERα was predominantly present in the cytoplasm, suggesting that deregulation of arginine methylases may have consequences in activation of ERα extranuclear actions [45]. Collectively, these emerging results suggest that ER extranuclear signaling has the potential to promote breast cancer cell migration and metastasis.

ERα **regulation of metastasis**

Metastases spawned by malignant tumors that have acquired increased invasiveness are responsible for almost all breast cancer-related morbidity and mortality. The majority of ERα-positive cells retain their ERα and respond positively to initial endocrine therapy for the treatment of advanced metastatic disease. Several recent studies have detected the presence of ERα expression in metastatic tumors [46-48]. A correlation between ERα-positive tumors and the development of bone metastasis has been observed clinically [49, 50]. Many metastatic tumors retain ERα. If primary tumors are ERα positive, greater than 80% of the lymph node metastases and 65–70% of distant metastases retain ERα [46, 47]. A clinical correlation has also

been reported between ERα-positive tumors and the development of bone metastasis [49, 50]. ERα signaling has also been shown to enhance lung metastasis [51]. In addition, ERα-mediated signaling has enhanced lung metastasis by promoting host-compartment response [51]. These emerging findings suggest that ERα signaling plays a role in metastasis.

ERβ regulation of cell migration and metastasis

ERβ, similar to ERα also functions as a transcription factor that mediates different physiological responses to estrogen signaling. However, the physiological consequences of ERβ-mediated transcriptional regulation are distinct from those of ERα [1]. A number of recent studies suggest that an increase in ERβ expression decreases cell proliferation and that ERβ has anti-proliferative (tumor suppressor) functions [52-54]. Reduced expression of ERβ was reported in invasive breast cancer [55] and ERβ expression is associated with less invasive and proliferating tumors [56]. Down regulation of ERβ is shown to promote epithelial to mesenchymal transition (EMT) in prostate cancer cells [57]. A recent study using breast cancer models cells provided evidence that ERβ expression was associated with less cell migration. Mechanistic studies indicated that ERβ affects integrin expression and clustering and consequently modulates adhesion and migration of breast cancer cells [58]. Collectively, the emerging evidence in various model cells (including ovary and prostrate) suggests that ERβ signaling may promote anti-migratory and anti-invasive responses; however, future studies using breast models are needed to further validate these findings.

Estrogen regulation of EMT

EMT constitutes the loss of hallmark structures and physiologic properties associated with the epithelia and the gain of new properties, including migratory and invasive growth patterns [59]. Loss of E-cadherin is a key initial step in the transdifferentiation of epithelial cells to a mesenchymal phenotype, which occurs when tumor epithelial cells invade the surrounding tissues [60]. Evolving evidence suggest that estrogen signaling can influence EMT and ERα signaling cross talk with several EMT regulators such as Snail and Slug. ERα directly binds to and regulates the promoter of metastasis tumor antigen (MTA) 3 that suppresses *Snail*, a gene implicated in EMT transition [61]. ERα down-regulates *Slug* transcription by the formation of a co-repressor complex involving HDAC1 (histone deacetylase 1) and N-CoR (nuclear receptor co-repressor) [62]. Estrogen promotes down-regulation of E-cadherin via transcriptional regulation by recruitment of corepressors such as scaffold attachment factor B [63]. Estrogen plays an important role in cytoskeletal rearrangements mediated by delocalization of E-cadherin [64]. Furthermore, a recent study found that E2 promotes reversible EMT-like transition as well as collective motility in ERα-positive cells [65]. Estrogen-regulated EMT is complex and is dependent on temporal expression patterns of MTA family members, cell adhesion-essential regulators and ER coregulators [66]. ERα signaling negatively regulates EMT by modulating MTA3 expression and thus promotes differentiation [61]. Collectively, these findings implicate that estrogen-mediated EMT depends on the cellular repertoire of ERα coregulators and EMT regulators, and that their cross talk has potential to differentially affect breast cancer progression, leading to metastasis via EMT changes.

Tumor microenvironment regulation of ER signaling

The metastasis signaling cascade is orchestrated through the activation of biochemical pathways that involve the tumor microenvironment. Stromal cells (fibroblasts, inflammatory cells and

endovascular cells) play important roles to create a supportive environment for tumor cell growth [67, 68]. Chemokines produced by stromal cells have potential to influence ERα-positive breast cancer progression to metastasis. The chemokine CXCL12/SDF-1 and its G-protein-coupled receptor CXCR4-mediated signaling pathways play important roles in the migration and invasion of breast cancer cells. Some evidence suggests that HER2-mediated breast tumor metastasis may involve HER2 and CXCR4 signaling pathway cross talk [69]. CXCR4 overexpression correlated with worse prognosis in patients, and constitutive activation of CXCR4 in poorly metastatic ER-positive MCF7 cells led to enhanced tumor growth and metastasis. The results from this study showed that enhanced CXCR4 signaling is sufficient to drive ERα-positive breast cancers to a metastatic and endocrine therapy-resistant phenotype via increases in MAPK signaling [70].

The intra-tumoral levels of estrogens and growth factors are regulated by the tumor-stromal interactions in the tumor microenvironment [71]. Cross talk between the tumor and stromal cells promote expression of aromatase, a key enzyme in E2 biosynthesis, resulting in intra-tumoral estrogen production in postmenopausal breast tumors [72]. Tumor-stromal cross talk regulates aromatase gene expression via the production of various factors such as COX2, tumor necrosis factor-α, interleukin-6 and interleukin-11 [71]. Tumor-stromal interactions also contribute to the expression of growth factors such as EGF and IGF-1, which activate the ERα through growth factor receptor cross talk, leading to ERα-positive breast cancer progression [73].

ER signaling components as potential biomarkers for predicting metastasis

ERα status is routinely used in the clinic for treatment selection; however, additional markers are urgently needed to predict metastasis. Considering the evolving significance of ERα coregulators

(SRC family members such as SRC-3/AIB1) in mammary tumor invasion and metastasis [74], SRC-3 status could be used as a diagnostic biomarker. Similarly, expression of the ER coregulator PELP1 is deregulated in metastatic breast tumors [37] and PELP1 protein expression is an independent prognostic predictor of breast cancer-specific survival and disease-free survival [38]. Since PELP1 plays a critical role in estrogen-mediated extranuclear signaling, these findings suggest that PELP1 could be used as a potential biomarker for predicting ER-driven metastasis. Several studies using various Src kinase inhibitors and dominant-negative mutants demonstrated that inhibiting c-Src activity decreased the metastatic potential of breast cancer cells [75]. Given the role of Src kinase in ER signaling, phospho c-Src is an attractive biomarker for predicting breast cancer metastasis in conjunction with other prognostic factors. Few recent preclinical studies using Src inhibitors confirmed the downstream target of Phos-Src and -FAK and could be possible diagnostic markers [76]. Because AKT signaling is implicated in invasive ductal carcinoma of the breast and implicated in ERα-mediated extranuclear actions leading migration/invasion, Phospho AKT (pAKT) status could be a potential biomarker in the prediction of therapeutic response in invasive ductal carcinoma of the breast [74]. Even though these emerging findings suggest ERα-signaling molecules as potential biomarkers, additional studies using a large set of human tumor samples are needed to clearly establish them as prognostic markers.

Therapeutic targeting of ERα signaling for blocking metastasis

The emerging significance of the ERα in the metastatic cascade indicates novel possibilities for therapeutic targeting of specific ERα signaling components that mediate migration, invasion and EMT. A large portion of metastases retain their ERα when the primary tumors are ERα-positive.

Several recent studies detected the presence of ERα and aromatase expression in metastatic tumors [46-48]. We envision that the therapies targeting ER signaling axis leading to metastasis are more suitable for early stage patients who have tumors that are amenable to biopsy and IHC analysis. Potential markers of ERα signaling that are implicated in metastasis (including kinases such as Src, AKT, and PI3K and coregulators such as PELP1, AIB1, and SRC-1) could be used in addition to traditional ERα status to identify this subset of patients.

Aromatase is recognized as a potent target in endocrine therapy for the treatment of postmenopausal breast cancers [73]. Because some metastases retain their ERα signaling, screening of patients with advanced breast cancer for expression of ERα, ER-coregulators and aromatase, may provide a rationale for the development of customized treatment of a subset of patients with ERα-positive and aromatase-positive cancer. These patients could be treated with an aromatase inhibitor (Letrozole) that ablates peripheral estrogen synthesis and ERα degraders/signaling blockers for their ERα-positive metastatic tumors.

Because ERα and ERβ have different physiological functions and have ligand binding properties that differ enough to be selective in their ligand binding, opportunities now exist for testing of novel ER subtype-specific, selective ER-modulators [77]. Several synthetic or novel natural compounds derived from plant materials have the potential to function as ERβ agonists [54, 78] and these compounds may have utility in augmenting ERβ tumor suppressive functions. If ERβ can hamper the regulation of ERα and inhibit the proliferation as well as affect the cross-talk with growth factors and their receptors, testing of ERβ agonist in combination with other endocrine therapies will provide a novel means to target ERα driven metastasis. Recent studies found a therapeutic efficacy using ERβ agonists in combination with aromatase inhibitors and

this strategy may be useful in treating aromatase inhibitor (AI)-resistant metastatic breast cancer [79].

ERα-positive metastasis has been associated with chemokine signaling through SDF-1-CXCR4. Therefore CXCR4 signaling is a rational therapeutic target for the treatment of ER-positive advanced breast carcinomas [70]. Integrin-linked kinase (ILK) is a nodal molecule in many molecular pathways that are implicated in cancer metastasis. Recent evidence suggests that ER extranuclear signaling utilizes the ILK axis [10]; therefore, ILK inhibitors such as QLT-0267 could be used to curb motility of breast cancer cells [80]. Since arginine methylation is implicated in ERα extranuclear signaling, blocking arginine methylases could be a possible therapeutic target. Compounds such as guanidine nitrogen-substituted peptides or the thioglycolic amide RM65 may be useful to block this pathway [81, 82]. SRC3/AIB1 is frequently amplified or overexpressed in human breast cancer and is implicated in breast cancer progression to advanced ERα-positive tumors. Mechanistic studies showed AIB1 over-expression activates the mammalian target of rapamycin (mTOR) and activation of mTOR pathway is critical for AIB1-driven tumorigenesis [83]. Recent studies suggest that mTOR inhibition and ER-targeted endocrine therapy may improve the outcome of the subset of patients with ER-positive breast cancers overexpressing AIB1 [84].

Emerging evidence that Src participates in ERα extranuclear actions and its wide deregulation in breast tumors suggests that it could be a potential candidate for treating ERα-positive metastasis [85]. The fact that Src can mediate interactions between the ERα and growth factor signaling pathways is of particular importance because cross talk between these pathways is implicated in activation of ERα extranuclear signaling leading to cell migration and invasion [10]. Further, the ability of the Src axis to promote local estrogen synthesis via aromatase

activation has potential to form an autocrine loop of ERα signaling leading to tumor cell proliferation and metastasis [86]. Thus blocking the Src axis could block ERα signaling at multiple fronts and thus reducing the ability of the ERα to promote metastasis. Recent studies found that inhibition of the Src family tyrosine kinases using inhibitors such as dasatinib can block ERα-mediated extranuclear actions leading to cell migration and invasion [10]. Therefore, it is tempting to speculate that combination of hormonal therapy with dasatinib, an orally available inhibitor of Src family tyrosine kinases that is currently approved for clinical trials to treat solid tumors [87-89], may be useful in curbing breast cancer metastases.

Conclusions/significance

The most deadly aspect of breast cancer is its ability to spread or metastasize. Recent mechanistic studies have increased our understanding and highlight a role of estrogen-induced rapid ERα extranuclear signaling in facilitating the metastatic process. This signaling pathway thus provides new targets for therapeutic intervention. During progression from tumorigenesis to invasion, tumor cells trigger signals that activate ERα extranuclear signaling pathways, leading to enhanced cell migratory functions and metastasis, thus ER extranuclear signaling represents an important target for metastatic control of ERα-positive tumors (Figure 2). Since multiple signaling pathways in addition to estrogen are involved in activating ERs, combination therapies using both endocrine and nonendocrine agents that block different pathways may have better therapeutic effects and may delay the development of estrogen-driven metastasis. Future studies identifying the molecular mechanisms of ERα signaling contributing to ERα-driven metastasis as well as examining the prognostic / diagnostic significance of ERα signaling components using

a larger sample size of tumors is warranted. Further, elucidation of the pathologic roles of ERα extranuclear signaling in metastasis will have important implications for development of novel breast cancer therapeutics and in the development of the next generation of selective ER modulators.

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Reference List

[1] Thomas C, Gustafsson JA. The different roles of ER subtypes in cancer biology and therapy. Nat Rev Cancer 2011 Jul 22;11(8):597-608.

[2] Lewis-Wambi JS, Jordan VC. Treatment of Postmenopausal Breast Cancer with Selective Estrogen Receptor Modulators (SERMs). Breast Dis 2005;24:93-105.

[3] Leary A, Dowsett M. Combination therapy with aromatase inhibitors: the next era of breast cancer treatment? Br J Cancer 2006 Sep;95:661-6.

[4] Utsumi T, Kobayashi N, Hanada H. Recent perspectives of endocrine therapy for breast cancer. Breast Cancer 2007;14(2):194-9.

[5] Steeg PS. Tumor metastasis: mechanistic insights and clinical challenges. Nat Med 2006 Aug;12(8):895-904.

[6] Stetler-Stevenson WG, Liotta LA, Kleiner DE, Jr. Extracellular matrix 6: role of matrix metalloproteinases in tumor invasion and metastasis. FASEB J 1993 Dec;7(15):1434-41.

[7] Friedl P, Wolf K. Tumour-cell invasion and migration: diversity and escape mechanisms. Nat Rev Cancer 2003 May;3(5):362-74.

[8] Rochefort H, Platet N, Hayashido Y, Derocq D, Lucas A, Cunat S, et al. Estrogen receptor mediated inhibition of cancer cell invasion and motility: an overview. J Steroid Biochem Mol Biol 1998 Apr;65(1-6):163-8.

[9] Sisci D, Aquila S, Middea E, Gentile M, Maggiolini M, Mastroianni F, et al. Fibronectin and type IV collagen activate ERalpha AF-1 by c-Src pathway: effect on breast cancer cell motility. Oncogene 2004 Nov 25;23(55):8920-30.

[10] Chakravarty D, Nair SS, Santhamma B, Nair BC, Wang L, Bandyopadhyay A, et al. Extranuclear Functions of ER Impact Invasive Migration and Metastasis by Breast Cancer Cells. Cancer Res 2010 May 11;70(10):4092-101.

[11] Zheng S, Huang J, Zhou K, Zhang C, Xiang Q, Tan Z, et al. 17beta-Estradiol Enhances Breast Cancer Cell Motility and Invasion via Extra-Nuclear Activation of Actin-Binding Protein Ezrin. PLoS One 2011;6(7):e22439.

[12] Giretti MS, Fu XD, De RG, Sarotto I, Baldacci C, Garibaldi S, et al. Extra-nuclear signalling of estrogen receptor to breast cancer cytoskeletal remodelling, migration and invasion. PLoS One 2008 May 21;3(5):e2238.

[13] Sanchez AM, Flamini MI, Baldacci C, Goglia L, Genazzani AR, Simoncini T. Estrogen receptor-alpha promotes breast cancer cell motility and invasion via focal adhesion kinase and N-WASP. Mol Endocrinol 2010 Nov;24(11):2114-25.

[14] Li Y, Wang JP, Santen RJ, Kim TH, Park H, Fan P, et al. Estrogen stimulation of cell migration involves multiple signaling pathway interactions. Endocrinology 2010 Nov;151(11):5146-56.

[15] Warner M, Nilsson S, Gustafsson JA. The estrogen receptor family. Curr Opin Obstet Gynecol 1999 Jun;11(3):249-54.

[16] Curtis HS, Couse JF, Korach KS. Estrogen receptor transcription and transactivation: Estrogen receptor knockout mice: what their phenotypes reveal about mechanisms of estrogen action. Breast Cancer Res 2000;2(5):345-52.

[17] Kumar V, Green S, Stack G, Berry M, Jin JR, Chambon P. Functional domains of the human estrogen receptor. Cell 1987 Dec 24;51(6):941-51.

[18] McKenna NJ, Lanz RB, O'Malley BW. Nuclear receptor coregulators: cellular and molecular biology. Endocr Rev 1999 Jun;20(3):321-44.

[19] McDonnell DP, Norris JD. Connections and regulation of the human estrogen receptor. Science 2002 May 31;296(5573):1642-4.

[20] Tsai MJ, O'Malley BW. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 1994;63:451-86.

[21] Barnes CJ, Vadlamudi RK, Kumar R. Novel estrogen receptor coregulators and signaling molecules in human diseases. Cell Mol Life Sci 2004 Feb;61(3):281-91.

[22] Collingwood TN, Urnov FD, Wolffe AP. Nuclear receptors: coactivators, corepressors and chromatin remodeling in the control of transcription. J Mol Endocrinol 1999 Dec;23(3):255-75.

[23] Kumar R, Gururaj AE, Vadlamudi RK, Rayala SK. The clinical relevance of steroid hormone receptor corepressors. Clin Cancer Res 2005 Apr 15;11(8):2822-31.

[24] Hall JM, McDonnell DP. Coregulators in nuclear estrogen receptor action: from concept to therapeutic targeting. Mol Interv 2005 Dec;5(6):343-57.

[25] Han SJ, DeMayo FJ, Xu J, Tsai SY, Tsai MJ, O'Malley BW. Steroid receptor coactivator (SRC)-1 and SRC-3 differentially modulate tissue-specific activation functions of the progesterone receptor. Mol Endocrinol 2006 Jan;20(1):45-55.

[26] Xu J, Qiu Y, DeMayo FJ, Tsai SY, Tsai MJ, O'Malley BW. Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. Science 1998 Mar20;279(5358):1922-5.

[27] Xu J, Liao L, Ning G, Yoshida-Komiya H, Deng C, O'Malley BW. The steroid receptor coactivator SRC-3 (p/CIP/RAC3/AIB1/ACTR/TRAM-1) is required for normal growth, puberty, female reproductive function, and mammary gland development. Proc Natl Acad Sci U S A 2000 Jun 6;97(12):6379-84.

[28] O'Malley BW. Molecular biology. Little molecules with big goals. Science 2006 Sep 22;313(5794):1749-50.

[29] Wang S, Yuan Y, Liao L, Kuang SQ, Tien JC, O'Malley BW, et al. Disruption of the SRC-1 gene in mice suppresses breast cancer metastasis without affecting primary tumor formation. Proc Natl Acad Sci U S A 2009 Jan 6;106(1):151-6.

[30] Han JS, Crowe DL. Steroid receptor coactivator 1 deficiency increases MMTV-neu mediated tumor latency and differentiation specific gene expression, decreases metastasis, and inhibits response to PPAR ligands. BMC Cancer 2010 Nov 16;10:629.

[31] O'Malley BW, Kumar R. Nuclear receptor coregulators in cancer biology. Cancer Res 2009 Nov 1;69(21):8217-22.

[32] Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. Cancer Res 2008 May 15;68(10):3645-54.

[33] Beavon IR. The E-cadherin-catenin complex in tumour metastasis: structure, function and regulation. Eur J Cancer 2000 Aug;36(13 Spec No):1607-20.

[34] Mazumdar A, Wang RA, Mishra SK, Adam L, Bagheri-Yarmand R, Mandal M, et al. Transcriptional repression of oestrogen receptor by metastasis-associated protein 1 corepressor. Nat Cell Biol 2001 Jan;3(1):30-7.

[35] Qin L, Liao L, Redmond A, Young L, Yuan Y, Chen H, et al. The AIB1 oncogene promotes breast cancer metastasis by activation of PEA3-mediated matrix metalloproteinase 2 (MMP2) and MMP9 expression. Mol Cell Biol 2008 Oct;28(19):5937-50.

[36] Qin L, Liu Z, Chen H, Xu J. The steroid receptor coactivator-1 regulates twist expression and promotes breast cancer metastasis. Cancer Res 2009 May 1;69(9):3819-27.

[37] Rajhans R, Nair S, Holden AH, Kumar R, Tekmal RR, Vadlamudi RK. Oncogenic Potential of the Nuclear Receptor Coregulator Proline-, Glutamic Acid-, Leucine-Rich Protein 1/Modulator of the Nongenomic Actions of the Estrogen Receptor. Cancer Res 2007 Jun 1;67(11):5505-12.

[38] Habashy HO, Powe DG, Rakha EA, Ball G, Macmillan RD, Green AR, et al. The prognostic significance of PELP1 expression in invasive breast cancer with emphasis on the ER-positive luminal-like subtype. Breast Cancer Res Treat 2009 Jun 3.

[39] Levin ER. Integration of the extranuclear and nuclear actions of estrogen. Mol Endocrinol 2005 Aug;19(8):1951-9.

[40] Harrington WR, Kim SH, Funk CC, Madak-Erdogan Z, Schiff R, Katzenellenbogen JA, et al. Estrogen dendrimer conjugates that preferentially activate extranuclear, nongenomic versus genomic pathways of estrogen action. Mol Endocrinol 2006 Mar;20(3):491-502.

[41] Finn RS. Targeting Src in breast cancer. Ann Oncol 2008 Aug;19(8):1379-86.

[42] Persad S, Dedhar S. The role of integrin-linked kinase (ILK) in cancer progression. Cancer Metastasis Rev 2003 Dec;22(4):375-84.

[43] Bjornstrom L, Sjoberg M. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. Mol Endocrinol 2005 Apr;19(4):833-42.

[44] Kumar R, Wang RA, Mazumdar A, Talukder AH, Mandal M, Yang Z, et al. A naturally occurring MTA1 variant sequesters oestrogen receptor-alpha in the cytoplasm. Nature 2002 Aug 8;418(6898):654-7.

[45] Teyssier C, Le RM, Sentis S, Jalaguier S, Corbo L, Cavailles V. Protein arginine methylation in estrogen signaling and estrogen-related cancers. Trends Endocrinol Metab 2010 Mar;21(3):181-9.

[46] Harrell JC, Dye WW, Allred DC, Jedlicka P, Spoelstra NS, Sartorius CA, et al. Estrogen receptor positive breast cancer metastasis: altered hormonal sensitivity and tumor aggressiveness in lymphatic vessels and lymph nodes. Cancer Res 2006 Sep 15;66(18):9308-15.

[47] Zheng WQ, Lu J, Zheng JM, Hu FX, Ni CR. Variation of ER status between primary and metastatic breast cancer and relationship to p53 expression*. Steroids 2001 Dec;66(12):905-10.

[48] Lu J, Li H, Cao D, Di G, Wu J, Sheng K, et al. Clinical significance of aromatase protein expression in axillary node negative breast cancer. J Cancer Res Clin Oncol 2007 Jun;133(6):401-9.

[49] Koenders PG, Beex LV, Langens R, Kloppenborg PW, Smals AG, Benraad TJ. Steroid hormone receptor activity of primary human breast cancer and pattern of first metastasis. The Breast Cancer Study Group. Breast Cancer Res Treat 1991 Mar;18(1):27-32.

[50] Wang J, Jarrett J, Huang CC, Satcher RL, Jr., Levenson AS. Identification of estrogen-responsive genes involved in breast cancer metastases to the bone. Clin Exp Metastasis 2007;24(6):411-22.

[51] Banka CL, Lund CV, Nguyen MT, Pakchoian AJ, Mueller BM, Eliceiri BP. Estrogen induces lung metastasis through a host compartment-specific response. Cancer Res 2006 Apr 1;66(7):3667-72.

[52] Speirs V, Carder PJ, Lane S, Dodwell D, Lansdown MR, Hanby AM. Oestrogen receptor beta: what it means for patients with breast cancer. Lancet Oncol 2004 Mar;5(3):174-81.

[53] Strom A, Hartman J, Foster JS, Kietz S, Wimalasena J, Gustafsson JA. Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. Proc Natl Acad Sci U S A 2004 Feb 10;101(6):1566-71.

[54] Nilsson S, Gustafsson JA. Estrogen receptors: therapies targeted to receptor subtypes. Clin Pharmacol Ther 2011 Jan;89(1):44-55.

[55] Skliris GP, Munot K, Bell SM, Carder PJ, Lane S, Horgan K, et al. Reduced expression of oestrogen receptor beta in invasive breast cancer and its re-expression using DNA methyl transferase inhibitors in a cell line model. J Pathol 2003 Oct;201(2):213-20.

[56] Jarvinen TA, Pelto-Huikko M, Holli K, Isola J. Estrogen receptor beta is coexpressed with ERalpha and PR and associated with nodal status, grade, and proliferation rate in breast cancer. Am J Pathol 2000 Jan;156(1):29-35.

[57] Mak P, Leav I, Pursell B, Bae D, Yang X, Taglienti CA, et al. ERbeta impedes prostate cancer EMT by destabilizing HIF-1alpha and inhibiting VEGF-mediated snail nuclear localization: implications for Gleason grading. Cancer Cell 2010 Apr 13;17(4):319-32.

[58] Lindberg K, Strom A, Lock JG, Gustafsson JA, Haldosen LA, Helguero LA. Expression of estrogen receptor beta increases integrin alpha1 and integrin beta1 levels and enhances adhesion of breast cancer cells. J Cell Physiol 2010 Jan;222(1):156-67.

[59] Nieto MA. The Ins and Outs of the Epithelial to Mesenchymal Transition in Health and Disease. Annu Rev Cell Dev Biol 2010 Oct 29.

[60] Schmalhofer O, Brabletz S, Brabletz T. E-cadherin, beta-catenin, and ZEB1 in malignant progression of cancer. Cancer Metastasis Rev 2009 Jun;28(1-2):151-66.

[61] Fujita N, Jaye DL, Kajita M, Geigerman C, Moreno CS, Wade PA. MTA3, a Mi-2/NuRD complex subunit, regulates an invasive growth pathway in breast cancer. Cell 2003 Apr 18;113(2):207-19.

[62] Ye Y, Xiao Y, Wang W, Yearsley K, Gao JX, Barsky SH. ERalpha suppresses slug expression directly by transcriptional repression. Biochem J 2008 Dec 1;416(2):179-87.

[63] Oesterreich S, Deng W, Jiang S, Cui X, Ivanova M, Schiff R, et al. Estrogen-mediated down-regulation of E-cadherin in breast cancer cells. Cancer Res 2003 Sep 1;63(17):5203-8.

[64] DePasquale JA. Rearrangement of the F-actin cytoskeleton in estradiol-treated MCF-7 breast carcinoma cells. Histochem Cell Biol 1999 Nov;112(5):341-50.

[65] Planas-Silva MD, Waltz PK. Estrogen promotes reversible epithelial-to-mesenchymal-like transition and collective motility in MCF-7 breast cancer cells. J Steroid Biochem Mol Biol 2007 Apr;104(1-2):11-21.

[66] Zhang H, Stephens LC, Kumar R. Metastasis tumor antigen family proteins during breast cancer progression and metastasis in a reliable mouse model for human breast cancer. Clin Cancer Res 2006 Mar 1;12(5):1479-86.

[67] Kalluri R, Zeisberg M. Fibroblasts in cancer. Nat Rev Cancer 2006 May;6(5):392-401.

[68] Allinen M, Beroukhim R, Cai L, Brennan C, Lahti-Domenici J, Huang H, et al. Molecular characterization of the tumor microenvironment in breast cancer. Cancer Cell 2004 Jul;6(1):17-32.

[69] Li YM, Pan Y, Wei Y, Cheng X, Zhou BP, Tan M, et al. Upregulation of CXCR4 is essential for HER2-mediated tumor metastasis. Cancer Cell 2004 Nov;6(5):459-69.

[70] Rhodes LV, Short SP, Neel NF, Salvo VA, Zhu Y, Elliott S, et al. Cytokine receptor CXCR4 mediates estrogen-independent tumorigenesis, metastasis, and resistance to endocrine therapy in human breast cancer. Cancer Res 2011 Jan 15;71(2):603-13.

[71] Simpson ER, Davis SR. Minireview: aromatase and the regulation of estrogen biosynthesis--some new perspectives. Endocrinology 2001 Nov;142(11):4589-94.

[72] Santen RJ, Santner SJ, Pauley RJ, Tait L, Kaseta J, Demers LM, et al. Estrogen production via the aromatase enzyme in breast carcinoma: which cell type is responsible? J Steroid Biochem Mol Biol 1997 Apr;61(3-6):267-71.

[73] Yamaguchi Y. Microenvironmental regulation of estrogen signals in breast cancer. Breast Cancer 2007;14(2):175-81.

[74] Park SS, Kim SW. Activated Akt signaling pathway in invasive ductal carcinoma of the breast: correlation with HER2 overexpression. Oncol Rep 2007 Jul;18(1):139-43.

[75] Sanchez-Munoz A, Perez-Ruiz E, Jimenez B, Ribelles N, Marquez A, Garcia-Rios I, et al. Targeted therapy of metastatic breast cancer. Clin Transl Oncol 2009 Oct;11(10):643-50.

[76] Jones RJ, Young O, Renshaw L, Jacobs V, Fennell M, Marshall A, et al. Src inhibitors in early breast cancer: a methodology, feasibility and variability study. Breast Cancer Res Treat 2009 Mar;114(2):211-21.

[77] Lo R, Matthews J. A new class of estrogen receptor beta-selective activators. Mol Interv 2010 Jun;10(3):133-6.

[78] Mersereau JE, Levy N, Staub RE, Baggett S, Zogovic T, Chow S, et al. Liquiritigenin is a plant-derived highly selective estrogen receptor beta agonist. Mol Cell Endocrinol 2008 Feb 13;283(1-2):49-57.

[79] Nair HB, Kirma NB, Ganapathy M, Vadlamudi RK, Tekmal RR. Estrogen receptor-beta activation in combination with letrozole blocks the growth of breast cancer tumors resistant to letrozole therapy. Steroids 2011 Jul;76(8):792-6.

[80] Kalra J, Warburton C, Fang K, Edwards L, Daynard T, Waterhouse D, et al. QLT0267, a small molecule inhibitor targeting integrin-linked kinase (ILK), and docetaxel can combine to produce synergistic interactions linked to enhanced cytotoxicity, reductions in P-AKT levels, altered F-actin architecture and improved treatment outcomes in an orthotopic breast cancer model. Breast Cancer Res 2009;11(3):R25.

[81] Spannhoff A, Machmur R, Heinke R, Trojer P, Bauer I, Brosch G, et al. A novel arginine methyltransferase inhibitor with cellular activity. Bioorg Med Chem Lett 2007 Aug 1;17(15):4150-3.

[82] Lakowski TM, 't HP, Ahern CA, Martin NI, Frankel A. N(eta)-Substituted Arginyl Peptide Inhibitors of Protein Arginine N-Methyltransferases. ACS Chem Biol 2010 Aug 26.

[83] Torres-Arzayus MI, Font de MJ, Yuan J, Vazquez F, Bronson R, Rue M, et al. High tumor incidence and activation of the PI3K/AKT pathway in transgenic mice define AIB1 as an oncogene. Cancer Cell 2004 Sep;6(3):263-74.

[84] Torres-Arzayus MI, Yuan J, DellaGatta JL, Lane H, Kung AL, Brown M. Targeting the AIB1 oncogene through mammalian target of rapamycin inhibition in the mammary gland. Cancer Res 2006 Dec 1;66(23):11381-8.

[85] Russello SV, Shore SK. SRC in human carcinogenesis. Front Biosci 2004 Jan 1;9:139-44.

[86] Rajhans R, Nair HB, Nair SS, Cortez V, Ikuko K, Kirma NB, et al. Modulation of in situ Estrogen Synthesis by PELP1: Potential ER Autocrine Signaling Loop in Breast Cancer Cells. Mol Endocrinol 2008 Mar 3;22:649-64.

[87] Huang F, Reeves K, Han X, Fairchild C, Platero S, Wong TW, et al. Identification of candidate molecular markers predicting sensitivity in solid tumors to dasatinib: rationale for patient selection. Cancer Res 2007 Mar 1;67(5):2226-38.

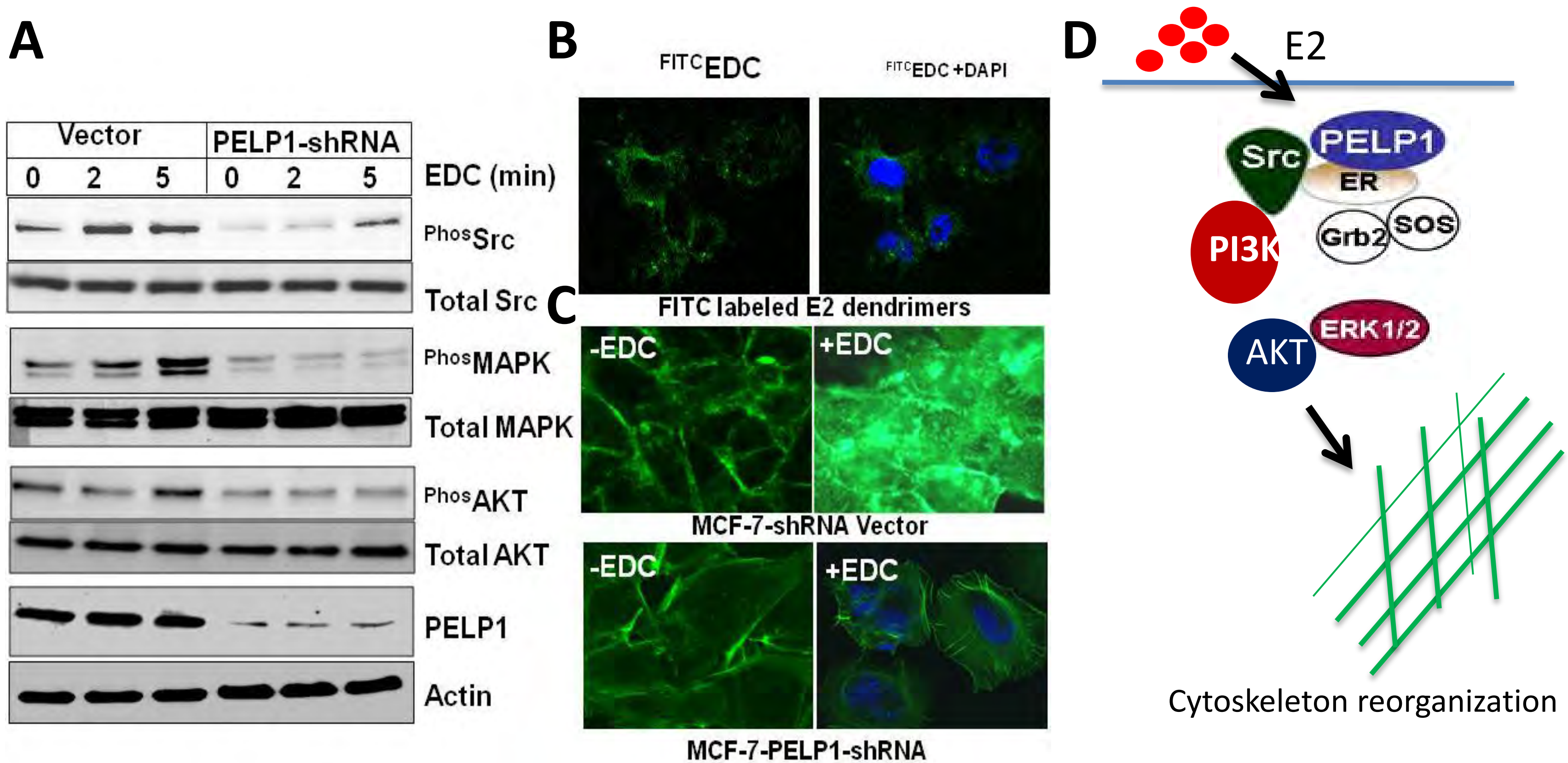
[88] Summy JM, Gallick GE. Treatment for advanced tumors: SRC reclaims center stage. Clin Cancer Res 2006 Mar 1;12(5):1398-401.

[89] Araujo J, Logothetis C. Dasatinib: a potent SRC inhibitor in clinical development for the treatment of solid tumors. Cancer Treat Rev 2010 Oct;36(6):492-500.

Figure Legends

Figure 1. ER-extranuclear signaling promotes actin reorganization via ER coregulator PELP1. *A*, MCF7 shRNA vector control and MCF7-PELP1-shRNA cells were cultured in 5% DCC serum containing medium treated with or without estrogen dendrimers (EDC). The activation of signaling pathways was analyzed by Western blotting of total protein lysates with phospho-specific antibodies. *B*, MCF7 cells were treated with FITC-labeled EDC and localization of EDC was analyzed by confocal microscopy. *Green; EDC; Blue, DAPI.* *C*, MCF7 or MCF7-PELP1-shRNA cells were treated either with E2 or EDC and the F-actin status was analyzed by phalloidin staining and visualized by confocal microscopy. *D*, Schematic representation of estrogen-mediated extranuclear signaling. Adapted from [10].

Figure 2. Schematic representation of hormonal regulation of metastasis. ERα-mediated signaling involves nuclear as well as extranuclear actions and growth factor signaling cross talk. Estrogen signaling has the potential to activate extranuclear signaling that activates several kinase cascades, which have potential to alter cytoskeleton, EMT and enhance cell migration. Deregulation of ERα-mediated signaling crosstalk will have implications in estrogen-mediated tumor progression to metastasis.



A
Vector
PELP1-shRNA
0 2 5 0 2 5
EDC (min)
PhosSrc
Total Src
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Total MAPK
PhosAKT
Total AKT
PELP1
Actin
B
FITCEDC
FITCEDC +DAPI
FITC labeled E2 dendrimers
C
-EDC
+EDC
MCF-7-shRNA Vector
-EDC
+EDC
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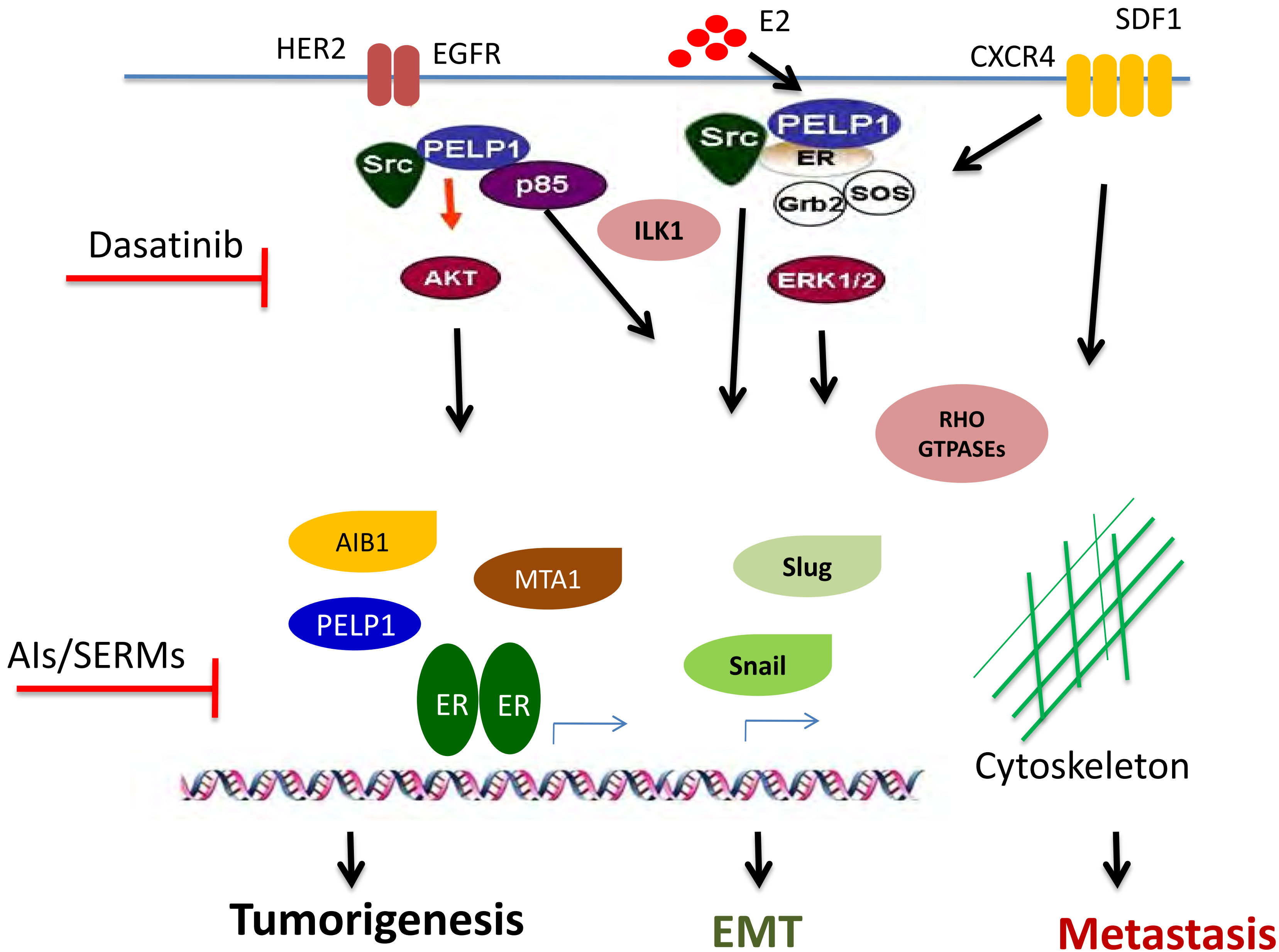
Cytoskeleton reorganization



HER2
EGFR
E2
SDF1
CXCR4
Src
PELP1
p85
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PELP1
ER
Grb2
SOS
ILK1
AKT
ERK1/2
Dasatinib
RHO GTPASEs
AIB1
MTA1
Slug
PELP1
Snail
AIs/SERMs
ER
ER
Cytoskeleton
Tumorigenesis
EMT
Metastasis